Characterization of Human Expired Breath by Solid Phase Microextraction and Analysis Using Gas Chromatography-Mass Spectrometry and Differential Mobility Spectrometry

by

William Merrick

S.B., Electrical Science and Engineering
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Characterization of Human Expired Breath by Solid Phase Microextraction and Analysis Using Gas Chromatography-Mass Spectrometry and Differential Mobility Spectrometry

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ABSTRACT

Breath analysis has potential to become a new medical diagnostic modality. In this thesis, a method for the analysis of human expired breath was developed using gas chromatography-mass spectroscopy. It was subsequently adopted for gas chromatography-differential mobility spectroscopy, a modality not previously applied to this problem. Tedlar bags and solid-phase microextraction were used for breath sampling and concentration prior to analysis. Four fiber coatings were evaluated with respect to selectivity and sensitivity; extraction time, gas chromatography temperature programming, and sample storage stability were explored for optimization. The method entails extraction and preconcentration with a polydimethylsiloxane-divinylbenzene coated fiber for 30 min at 37°C, and extraction profiles for several compounds demonstrate competitive adsorption. 120 compounds were identified in breath with response variability between 23 - 117% about mean values. Feasibility of differential mobility spectroscopy for breath analysis was established, and this method will be the basis for future investigations on the diagnostic potential of breath analysis.

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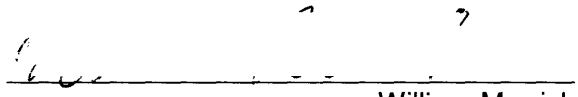
Publication of this thesis does not constitute approval by Draper of the sponsoring agency of the findings of conclusions contained therein. It is published for the exchange and stimulation of ideas.



William Merrick

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1 INTRODUCTION

1.1 Overview of Research

Medical diagnostics are an important and growing field in healthcare. While physicians diagnose disease based on symptoms, it is not always possible to determine the underlying cause or genetic and complicating factors that may govern the best course of treatment for a particular person. Thus, for many diseases, patients receive the same treatment regardless of the specific parameters that make their case unique. As we address this issue and develop a more personalized medical approach, there will be an increased demand on diagnostic tools to guide the direction of treatment. Additionally, diagnostics will be expected to provide more specific answers in a more timely manner. In areas such as cardiovascular disease, cancer, and kidney disease, early detection and quick response have been proven to significantly improve the outcome for patients who might otherwise develop fatal conditions if treatment is prevented or delayed. It is evident that rapid, sensitive diagnostics will play a critical role in delivering state-of-the art medical care in the future.

A new diagnostic modality that has tremendous potential is exhaled breath analysis. With false positives by screening tools and increased testing frequency with more specialized medical areas, the potential cost effectiveness and diagnostic capability of breath analysis can no longer be overlooked. Dozens of breath compounds show promise as markers for disease diagnosis and management. While some researchers have elaborated the diagnostic potential of such markers, there has been only modest technical or clinical development to date [1, 2].

The majority of breath analysis research has used Gas Chromatography (GC) and Mass Spectrometry (MS). The techniques employed have been limited by variability in ambient gas measurements and water saturated samples [3]. Effective breath analysis has previously been difficult due to the presence of only trace volatile compounds. Early methods for concentration can not be easily scaled for large studies or practical application [3]. Furthermore, past techniques have overlooked potentially relevant information in breath by focusing on only specific compounds. This goal of this project is to develop a method for breath analysis using novel technologies to address the above limitations. This new system will combine Solid Phase Microextraction (SPME) for sample concentration to address the detection and scalability difficulties; Differential Mobility Spectrometry (DMS), which can perform highly sensitive detection, is cost-effective, and has been miniaturized making it clinically applicable; and a data analysis technique based on cutting edge bioinformatics, which will permit analysis of the entire breath profile.

Investigators have demonstrated that specific breath compounds like pentane and isoprene are markers for reperfusion injury and can be elevated in ischemic and inflammatory heart disease [4-7]. This project will form the basis for production of a simple breath analysis device capable of diagnosis of acute cardiac distress patients in a clinical setting and that can be administered by minimally-trained personnel. The research presented here represents the initial phase of this project and is focused on the development and optimization of a dynamic breath sampling method. For practical reasons, method development was achieved using a gas chromatography-mass spectroscopy (GC-MS) system and subsequently implemented with the novel gas chromatography-differential mobility spectroscopy (GC-DMS) system to investigate

feasibility. Both systems were utilized to perform preliminary analytical work to study the normal variations in human breath profiles.

1.2 Breath Analysis Background

Breath analysis has garnered a great deal of interest in recent medical science because it offers a noninvasive window into normal metabolic pathways and has the potential to illustrate how these pathways are altered in disease [8-13]. Physicians have known for centuries that some diseases can be diagnosed simply by characteristic odors of patients' breath. For example, a sweet, fruity breath odor (acetone) is characteristic of poorly controlled diabetes mellitus, a fishy smell (dimethyl sulfide) accompanies advanced liver disease, and odors of urea, a main component in urine, are very common with patients suffering from kidney failure [1].

As early as 1927, Bogen [14] demonstrated the feasibility of using measurements of breath alcohol content to monitor blood alcohol levels. However, it required almost three decades to develop a device for practical use [15, 16]. Although subsequent support has been provided for the alcohol breath test, there was originally disagreements between scientists, governments, and law enforcement stemming from performance variability of the device and a lack of evidence for the underlying physiological mechanisms [17]. Two basic problems have hindered the advancement of breath diagnostics: lack of physiological understanding and inadequate analytical technology. Advancements in both areas should now allow the field of breath analysis to make substantial strides.

Breath exhalate is largely composed of nitrogen, oxygen, carbon dioxide, water, and inert gases. While these compounds have proved useful in diagnosis and monitoring of many disease conditions [18-20], it is the remaining fraction of human breath that

represents the most valuable part for diagnostic purposes [3]. This remaining fraction is made up of numerous volatile organic compounds (VOCs), each present in nanomolar (ppb to ppt) concentrations in the breath [3]. In contrast to highly abundant breath chemicals like nitrous oxide, volatile organic compounds mainly originate from the blood and can therefore provide insights into different biochemical processes in healthy and diseased individuals. VOCs derive from metabolic pathways and partition from the blood via the alveolar membrane into the alveolar air. The kinetics of VOCs can be approximated according to substance solubilities, implying that the concentrations measured in breath are related to concentrations in blood [21]. Breath analysis can be used diagnostically because aberrations in the concentrations of certain compounds have been associated with various diseases or altered metabolism [22].

The age of modern breath analysis began in 1971 when Pauling *et al.* discovered that normal human breath contained low, but measurable, concentrations of several hundred different VOCs [23]. The average human breath sample contains more than 200 different volatile organic compounds and there have been more than 3000 different VOCs observed in breath [24]. Most of these trace compounds are of an exogenous origin, but it is the measurement of endogenous markers that are most important for diagnostic purposes. While there exists a great deal of variation in the type and quantity of VOCs, only a few are consistently observed across multiple subjects [24]. Many of the most commonly occurring VOCs are derived from well understood metabolic pathways, and in disease, these VOCs have been shown to be elevated as much as ten fold. It is important to note that some chemicals may exist at levels below the detection capability of GC-MS, and a more sensitive detector could provide increased opportunities to recognize disease biomarkers. Moreover, we may discover that what defines a disease

is more than a simple chemical concentration threshold. Potentially, it could be relative ratios of a complex panel of chemicals that are definitive for disease detection in breath.

Some of the most well documented compounds are described here to illustrate the type of knowledge we now have about the origins and composition of breath. Isoprene (2-methylbutadiene-1, 3), a potential marker in acute cardiac distress, is frequently found in human breath. It is thought to be derived from the mevalonic acid pathway of cholesterol synthesis [25], logically following from the fact that cholesterol is often elevated in patients with cardiovascular disorders (Figure 1).

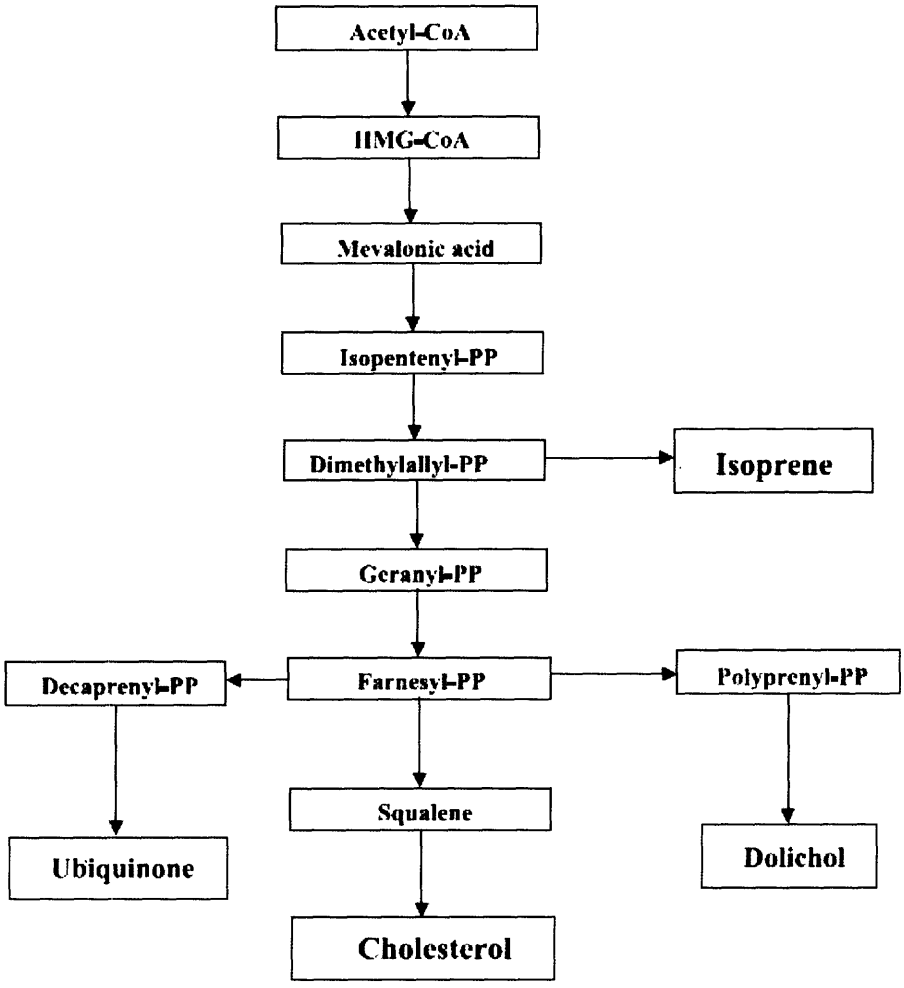

Figure 1: Biochemical pathway of isoprene generation: metabolism of mevalonate [3].

Acetone, an abundant VOC in human breath, is produced in the liver from the decarboxylation of acetoacetate, an intermediary in the breakdown of excess Acetyl-CoA. Excess Acetyl-CoA results from gylcolysis in response to glucose metabolism in diabetes [26]. Alkanes, like pentane and ethane, have been shown to arise from the oxidative degradation of polyunsaturated fatty acids [27]. Oxygen free radicals acting on omega-3 fatty acids such as 9,12,15-linolenic acid produces ethane, whereas pentane is the product of peroxidation of n-6 polyunsaturated acids such as 9,12,15-linolenic and arachidonic acid (Figure 2)[28] .

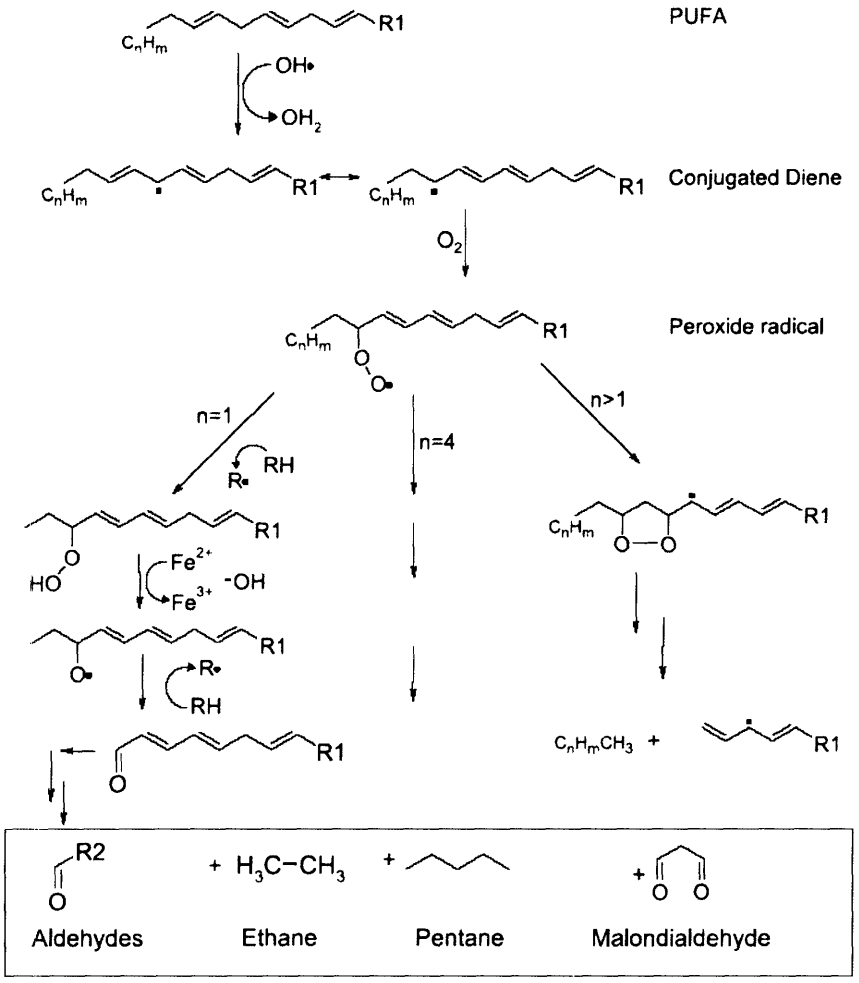

Figure 2: Free radical-mediated lipid peroxidation: possible reactions and reaction products [3].

Breath compounds are present in concentrations in the nM to pM range [29]. This can also be expressed as the number of molecules in dilute air, so concentrations are found at parts-per-billion (ppb) and parts-per-trillion (ppt) levels. To improve detection sensitivity and precision for these compounds, preconcentration may be necessary before the sample can be analyzed with new or standard detectors, such as gas chromatography-mass spectroscopy (GC-MS). Many techniques including chemical, adsorptive binding, sorbent traps, and cryofocussation have been developed for preconcentration of exhaled breath [30-32]. However, sample preparations using these techniques are tedious, time-consuming, and impractical for in-hospital breath analysis. Additionally, because of the high content of water and carbon dioxide in human breath, a pre-concentrating method that excludes these substances may also aid analysis.

Solid Phase Micro-Extraction (SPME), developed by Pawliszyn, *et al.* [33], has gained popularity in chemical analysis because it is fast, inexpensive, and a relatively efficient and effective method for sampling volatile organic compounds [28, 34]. SPME has been applied widely to the quantitative determination of specific VOCs in human breath such as ethanol, acetone and isoprene. The types of compounds concentrated by SPME depend on the specific phase material chosen. There are a number of SPME phases available commercially and many more can be customized for individual purposes. Polydimethylsiloxane (PDMS) is a standard liquid phase fiber with affinity to polar compounds. For volatile extractions in human breath, mixed phase fibers have been shown to be the best performing [28]. A blend with PDMS and divinylbenzene (DVB) and one with DVB and a proprietary polyethyleneglycol called Carbowax© are examined in this thesis.

1.3 Gas Chromatography- Mass Spectroscopy

Gas Chromatography-Mass Spectroscopy (GC-MS) is one of the most widely used and powerful tools for analytical chemistry and breath analysis. Although imprecision with GC-MS instrumentation makes it a complex system to work with, it is a proven technology for volatile chemical identification. Standardization and refinements have been made over the last few decades that have resulted in more reproducible analysis.

GC-MS represents a device that can separate chemical mixtures into individual components and then detect mass of those components. Sample flows through the chromatographic column where the sample is separated according to the chemical properties of the column. The various sample components emerge from the column into a chamber where components are ionized through electron impact. A collimated beam of electrons impacts the sample resulting in ionization and fragmentation of the molecule. In quadrupole mass spectroscopy, ion masses travel through a scanning electric field, which separates the positively charged particles by mass. After separation, mass fragments enter a detector which amplifies the signal and catalogs the charge and mass of the particle in a quantity called the mass-to-charge ratio (m/z). At each instance of time, mass spectra from all the detected masses are recorded (Figure 3). Collected data consists of chromatographic retention times, signal intensity, and mass-to-charge ratio measurements. Signal representation is usually in the form of total ion chromatograms (TICs) where the abundances of all ions at a particular time have been summed (Figure 4).

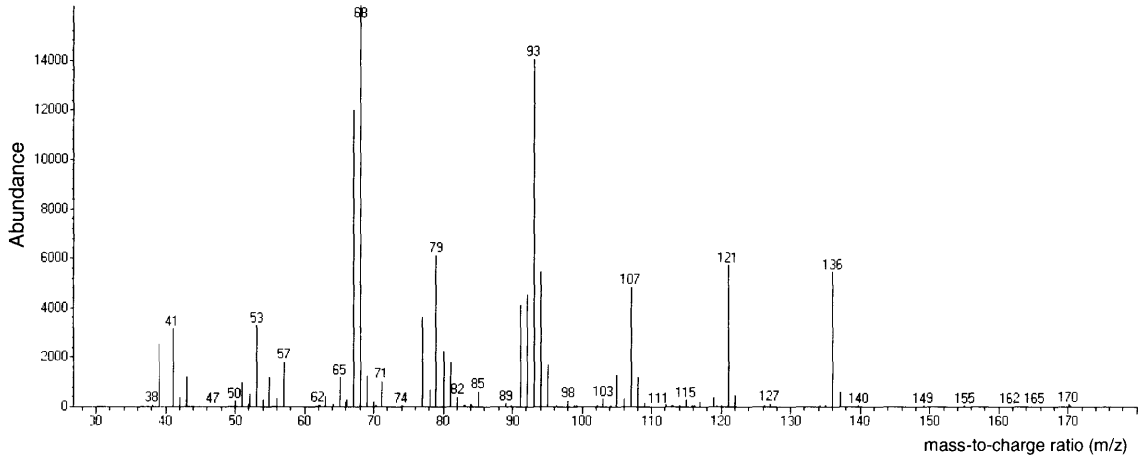

Figure 3: Mass spectrum of Limonene

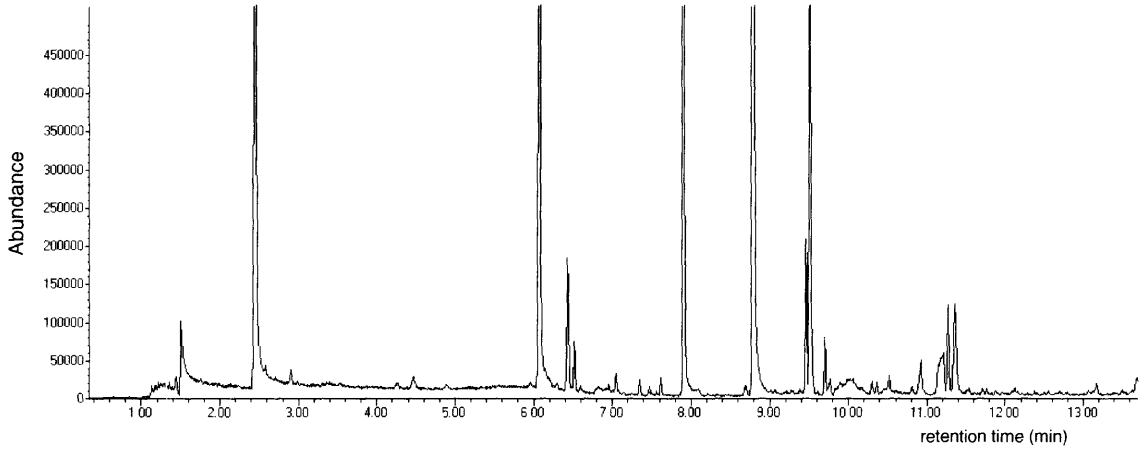

Figure 4: Total Ion Chromatogram (TIC) from Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS can be used for both qualitative and quantitative sample detection. Qualitative identification of compounds is based on mass spectral patterns since each compound has a unique fragmentation pattern. This type of analysis looks at intensities of particular masses as a function of time, and with specialized algorithms can match the unknown spectra to one in a library of several thousand known mass spectra. Component retention time (timing information based primarily on properties of the compound and chromatographic column) can be used to locate known compounds in the TIC and

enable quantification. Quantification is based on the peak area from mass chromatograms, or in some cases, by using selected ion monitoring.

1.4 Differential Mobility Spectroscopy (DMS)

Differential Mobility Spectroscopy (DMS) can be used for chemical and biological detection by measuring the differential mobility of ions in the sample mixture [35-42]. Analyte sample particles are ionized and filtered according to their mobility in high-amplitude radiofrequency electric fields. Ion mobility is a property dependent on the ion's charge, mass, and volume, as well as the applied electric field and gaseous environment. Sample classification with differential mobility spectroscopy makes use of the nonlinear relationship between ion mobility and field strength, where variable electric fields are applied to the sample to produce separation [43].

After combining with a carrier gas, a gas sample enters the sensor where it is ionized by a radioactive or ultraviolet source. In the DMS, ions are carried by a stream of nitrogen gas into a filter region between two charged plates. The plates have a constant radiofrequency (RF) electric field applied to them allowing for enhanced separation over ion mobility spectroscopy (IMS), which makes use of a constant DC voltage electric field. In DMS, the periodicity of the RF fields causes ions to have variable mobility. As a result, ions travel in an oscillatory fashion with a bias towards the upper or lower plate [38] (Figure 7). An additional DC compensation voltage is applied between the plates which can correct for the bias in the ion's path. The mobility of the ion is relative to the applied DC compensation voltage. Therefore, only compensated ions are able to traverse the entire length of the filter chamber and collide with a Faraday detector. Uncompensated ions fail to reach the detector and are instead scattered according to their bias towards either filter electrode, neutralized, and carried out of the system via carrier gas. By

sweeping through a range of compensation voltages over a short duration, DMS can simultaneously detect the diversity of ion species present in a sample. Constructing an interface with gas chromatography is simple. Similar to a mass spectrometer, the ion information provided by the DMS is enhanced by the additional dimension of information provided by the gas chromatogram [44].

Figures 5 and 6 provide examples of signal DMS signal representation. Figure 5 shows spectra with time on the x-axis, compensation voltage on the y-axis, and detected signal amplitude represented by color intensities. The representation in Figure 6 is called "Total Ion Abundance" and is similar to TIC. Here, the abundances of all compensation voltages are summed at each instant in time.

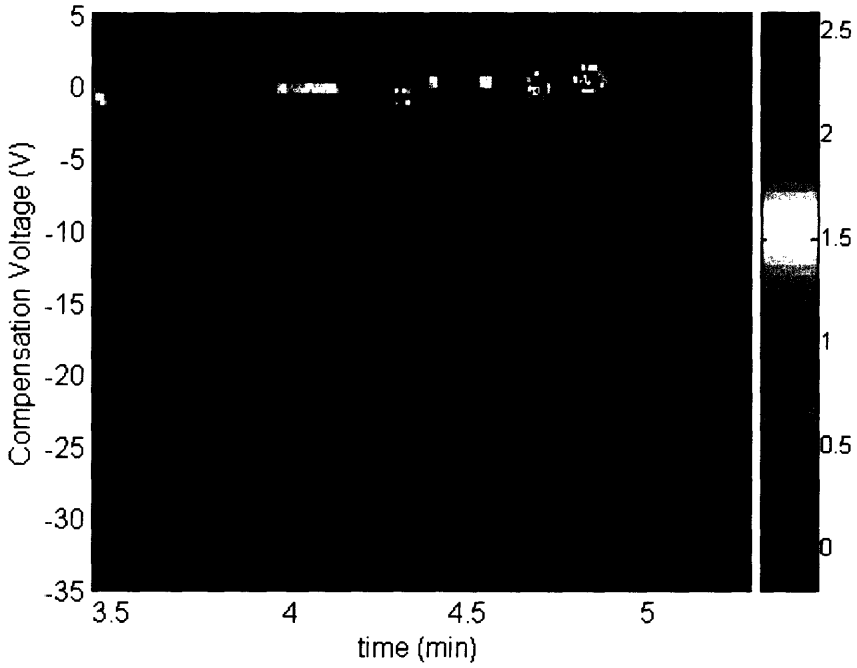

Figure 5: Example of Differential Mobility Spectra

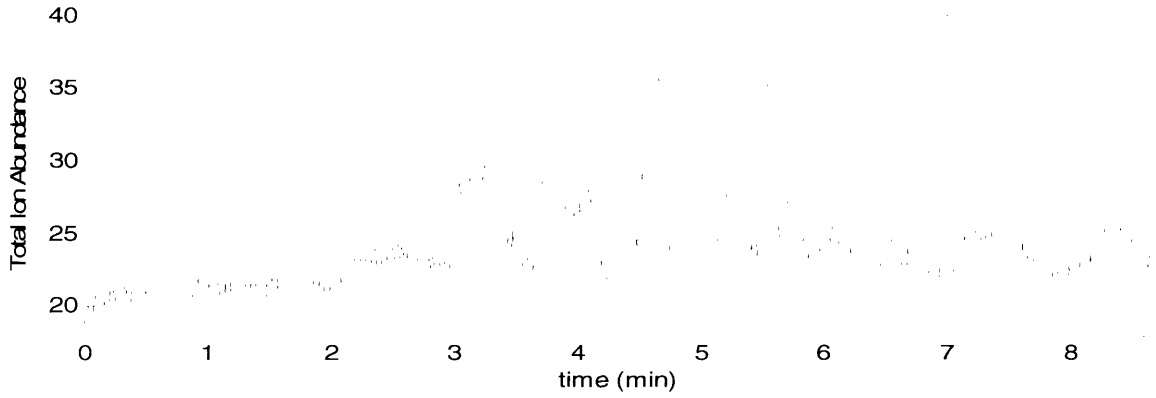

Figure 6: Total Ion Abundance (TIA) from Gas Chromatography-Differential Mobility Spectroscopy (GC-DMS)

Draper Laboratory has developed a sensitive, inexpensive differential mobility spectrometer for use in chemical and biological detection [38, 45]. The technology was licensed and further developed by Sionex Corp. (Waltham, Mass.). This sensor has superior sensitivity and detection resolution down to the parts-per-trillion range [37]. As a result of miniaturization, this sensor is able to be used in practical applications where mobile or inexpensive detection is sought (Figure 7).

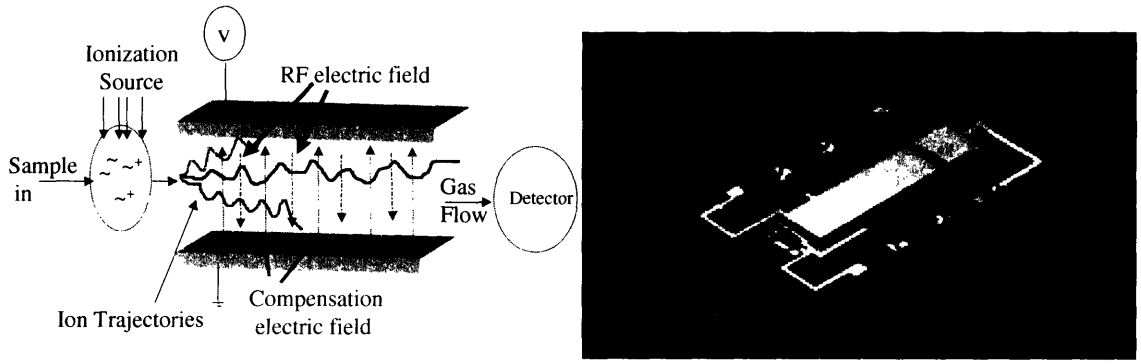

Figure 7: Ion motion between DMS filter electrodes showing complete passage of compensated ions (left). MEMS device for DMS developed by Sionex Corporation [38].

A DMS sensor coupled with a pyrolyzer for sample introduction has been used to successfully detect and identify bacteria spores as well as distinguish between related

bacteria and bacteria of different species [42, 45]. DMS has also been used with headspace sampling and gas chromatography for the detection of volatile organic compound fingerprints above bacterial headspace [41]. This research represents the first attempt to couple Gas Chromatography and Differential Mobility Spectroscopy (GC-DMS) with Solid Phase Microextraction for breath sample introduction.

1.5 Data Analysis Techniques

The ultimate goal of this research is to develop a methodology to analyze breath using Differential Mobility Spectroscopy. Unlike Gas Chromatography-Mass Spectroscopy, a library of compounds and their resulting spectra has not been developed for DMS detectors given that it is a relatively new technology. Furthermore, the spectra produced with this technology are based on nonlinear principles. So, although there may be well validated spectral patterns for compounds A and B, mixtures of these two compounds are not always expected to produce a signal that is simply the superposition of their independent signals. For a library-based analytical approach to be used, a library of all compounds and all combinations of compounds would have to be developed; this is impractical due to resource constraints. However, for data analysis of DMS spectra, our group has utilized chemometric techniques and pattern recognition approaches, specifically principal component analysis and genetic algorithm classification [41, 46].

For novel clinical applications, genetic algorithm-based pattern recognition has shown promise as a tool for DMS data analysis. In this work, we use a commercially available version of this algorithm, known as ProteomeQuest® (Correlogic Systems, Bethesda, MD).

This algorithm is particularly powerful because while other research focuses on single compounds, ProteomeQuest® determines biomarkers that account for the complex breath signatures that may be crucial in disease detection. ProteomeQuest® has been effectively used for pathogen detection and the classification of bacteria [41]. This pattern recognition technique identifies invariant biomarker features in the signal that are less susceptible to normal biological variability and background noise. The method development approach described in this paper will focus on optimizing signals for pattern recognition. Efforts will be focused on feature resolution and noise reduction, which have been shown to improve analysis performance.

1.6 Summary of Research

There were two purposes of the research carried out in this thesis. The first was method development for the both GC-MS and GC-DMS systems. The second goal was to perform preliminary analysis of the resulting breath signals from each system.

Since the combination of gas chromatography and differential mobility spectrometry has not been used for the analysis of breath, a GC-MS system was used in parallel for development and optimization of GC-DMS method parameters. GC-MS is well suited for comparison as previous studies have established methods for breath analysis. While not uniformly accepted, these methods provide a basis with which to compare our novel techniques. GC-MS also provides standard analytical tools and techniques to monitor the resulting breath signals which can provide quantitative guidance for method optimization.

Initially, we wanted to use this method development as an opportunity to identify the variety of compounds in breath and determine which components of the signal are derived from breath and which result from background. The next group of experiments focused on the application of solid phase microextraction to breath. Although SPME is a very powerful extraction technique, the choice of polymer coating and extraction conditions can significantly impact the ability to collect quality breath samples. Therefore, experiments were developed to answer the following questions: what type of SPME coating should be used, how long should the fiber be exposed to breath, and at what temperature should extraction take place? The separation of compounds in the breath sample is the responsibility of the gas chromatographic column. The temperature and the rate of change in temperature impact the performance of this step. The temperature profile of the oven housing the column was optimized for maximum signal resolution and minimum noise. The final experiment of method development aimed to determine the stability of breath samples stored for different durations. This was important as sample storage will be a necessary part of future applications where breath will be collected in the field and analyzed in the laboratory.

The second type of experiments focused on characterization of normal breath. We examined the normal variability in the composition of breath from a single individual as well as the variability between multiple subjects. We performed quantitative analysis to determine numerical similarity between samples using standard analytical metrics. This work was carried out on both the GC-MS and GC-DMS and represented the first application of an optimized breath analysis method for the GC-DMS system.

2 MATERIALS AND METHODS

As discussed previously, there are two purposes of the experiments conducted in this study: method development for breath collection and analysis and signal characterization. The method development experiments were set up to explore and optimize system parameters to provide the most robust and reproducible breath signal possible. Methods are described here for sample collection and analysis with SPME-GC-MS and SPME-GC-DMS systems. Emphasis is placed on exploring parameters that improve breath signal resolution and signal-to-noise ratios. Signal resolution can simply be thought of as how well individual breath compounds are separated from one another in the spectra. Breath is such a complex sample that it can be difficult to get rid of overlapping component signals in both of our evaluated spectral techniques.

As a starting point, method parameters for breath analysis were derived from literature [28, 34, 47, 48]. Other researchers have demonstrated success using SPME for breath analysis. However, there is no clear consensus on most appropriate SPME coating for this application. One of the probable reasons for this is that the majority of studies applying SPME to breath analysis have evaluated the performance of coatings with respect to a particular VOC or category of compounds. Therefore, we first sought to determine the fiber that yielded highest sensitivity and selectivity for the greatest number of breath compounds. Optimization of extraction conditions followed as extraction parameters should be determined experimentally when new combinations of polymer coating and sample are investigated. Extraction time was analyzed to determine the adsorption duration that would provide highest sensitivity to breath VOCs.

Gas chromatography determines separation characteristics of compounds in the detected signal. Therefore, GC temperature programs were developed and evaluated for signal resolution and noise levels. The process was performed iteratively and attempts were made to improve both performance measures with modification to temperature profile parameters. Finally, the effects of storage on sample stability were investigated to determine reasonable limitations on the acceptable time between sample collection and analysis.

Prior to starting a full scale clinical trial, it is difficult to evaluate whether the breath analysis approach described in this thesis will be valuable for disease diagnosis. Subject sampling in this study only looks at healthy individuals – completely missing cohorts that promise to have biomarkers for disease detection. Our goal in this limited study is to apply breath analysis methods to a novel detection system and characterize breath signal variability in normal populations. This provides us with a baseline evaluation of breath signals and allows us to move forward and plan a clinical trial to identify biomarkers in diseased patients. Table 1 outlines all of the experiments conducted as a part of this thesis. It provides information regarding the variables examined under each experiment and the number of samples analyzed.

Table 1: Summary of experiments

	number	sample	SPME	additional notes
Method development				
baseline breath & bag cleaning protocol				
	3	ambient air	PDMS/DVB	
	3	unused bag	PDMS/DVB	
	3	breath	PDMS/DVB	
	3	cleaned bag	PDMS/DVB	
SPME coating selection				
	3	breath	CAR/PDMS	
	3	breath	CW/DVB	
	3	breath	PDMS/DVB	
	3	breath	DVB/CAR/PDMS	
GC-MS settings				
	5	breath	PDMS/DVB	oven temperature profile varied
extraction time				
	18	breath	PDMS/DVB	extraction times = {1, 10,20, 30, 40, 120} min
storage stability				
	18	breath	PDMS/DVB	storage times = {0.5, 6, 12, 24, 48, 72} hrs
Chracterization of normal breath				
single subject				
	10	breath	PDMS/DVB	
multiple subjects				
	7	breath	PDMS/DVB	

2.1 Breath Sampling and Collection

2.5 L Tedlar® gas sampling bags (Welch Fluorocarbon, Dover, NH) were chosen for breath sample collection and storage. Tedlar, a polyvinyl fluoride film, is relatively non-reactive, durable, and less diffusive than other films (e.g. Teflon®) making it well suited for breath sampling applications [34]. Prior to first use, new sample bags were flushed with pure nitrogen for a minimum of 10 minutes. Breath from subjects was collected via a disposable, polyethylene mouthpiece (Allegiance Healthcare Corp., McGaw Park, IL). A special Tedlar lay-flat-tube was fitted to bags. The tube is open when the mouthpiece is inserted but seals and prevents sample escape when the mouthpiece is removed. Two Teflon coated polypropylene septa were added for easy sample extraction (Figure 8).

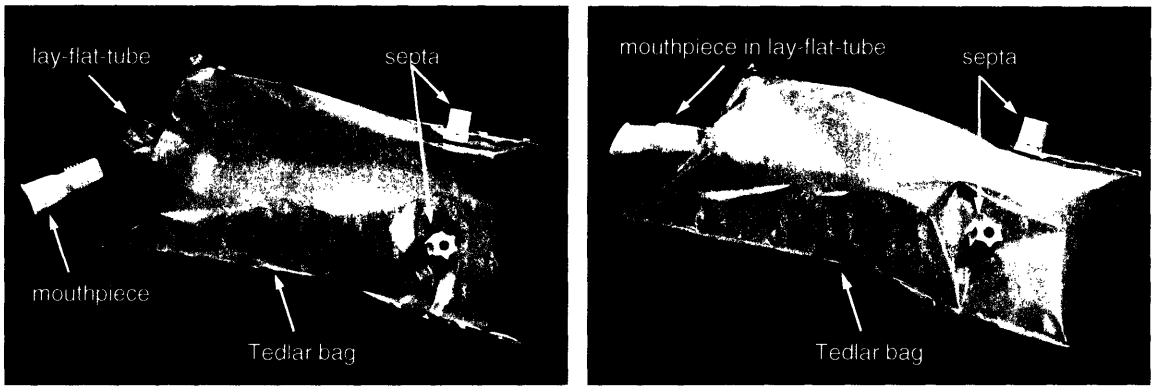

Figure 8: Breath sampling apparatus

In breath analysis, it is alveolar air that is most physiologically informative. Alveolar air is defined as the volume that equilibrates with gas concentrations in the blood. However, the technique for achieving alveolar air is not standard across the field although the method of collection can have an appreciable influence on the results of breath testing [3]. An exhalation is commonly divided into an initial (~ 150 ml) volume of dead space and the remainder is thought to be alveolar air. Two basic collection techniques are found in the literature, mixed expiratory sampling and alveolar sampling, which was used in this research [3]. Alveolar sampling requires elimination of dead space gas before sample collection. By sampling with this method concentrations of endogenous VOCs are two to three times higher than those in mixed expiratory air as the samples are not diluted with dead space gas. Furthermore, alveolar breath samples have less contamination [31, 49]. Figure 9 shows the three phases of exhalation. The goal of alveolar sampling is to collect exhalate only in phase III.

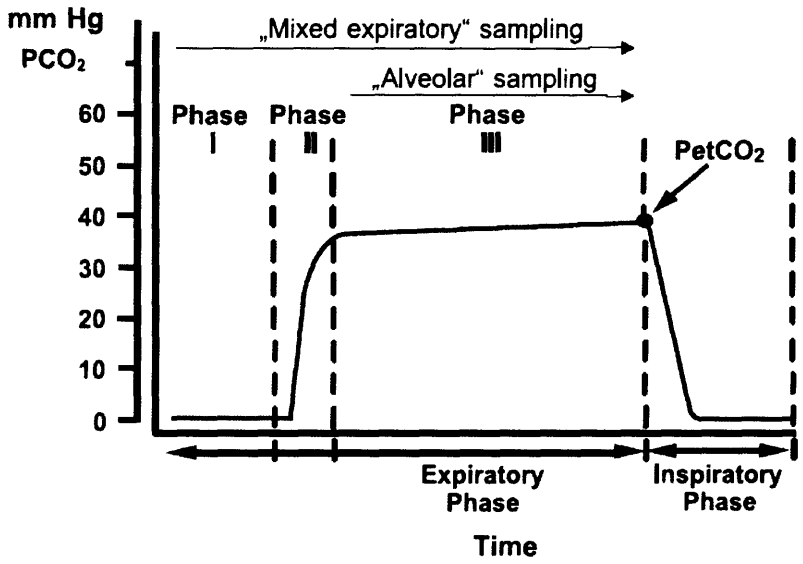

Figure 9: Schematic of a normal capnogram and typical modes of sampling [50]

In order to standardize the breath samples as much as possible, subjects were instructed to breath normally for at least five breaths. On the following breath they were asked to hold their breath for 5 sec and exhale to the end of their normal tidal volume. The technique of breath holding has been shown to improve the quality of alveolar sampling [51]. A breath sample was then collected by forced exhalation of the remaining lung volume into the sampling apparatus as long as it was comfortable. This has been determined to be best technique for non-invasive alveolar breath sampling [51]. Subjects were instructed to repeat these steps until the bag is approximately 80% filled.

Samples were stored at room temperature and analyzed within 30 min from collection, unless otherwise noted. Collected samples were incubated at 37°C (preheating) for 5 min before concentration with Solid-Phase Microextraction (SPME). SPME assemblies consist of a coated fiber and a manual holder (Supelco, Bellefonte, PA) (Figure 10). With the sample maintained at 37°C, the SPME fiber assembly is exposed to the sample for 30 min. The length of exposure is called the extraction time, and it was one of the parameters optimized as part of method development.

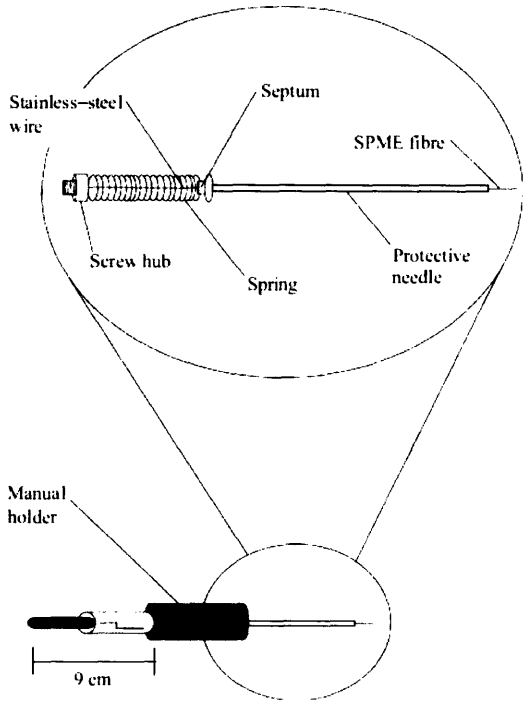

Figure 10: Schematic of solid phase microextraction assembly [52]

Prior to first use, SPME fibers were conditioned in the GC inlet following manufacturer instructions. Before each analysis run they were conditioned at 250°C for 10 min. Optimized extraction parameters were determined to be 30 min at 40°C. Validation of these parameters is discussed in further detail in Sections 3.2 and 3.3. The SPME fiber containing target VOCs was inserted into the GC inlet and thermally desorbed for 5 min at 250°C in carrier gas with flow rate of 2.0 ml/min. During SPME fiber desorption, the chromatographic column was held at 50°C. Desorbed volatiles were carried by helium gas to the column opening mounted at the base of the inlet where they partitioned into the liquid phase of the column film. Following the initial 5 min, the inlet purge was opened, the oven temperature program began, and the gas flow rate increased forcing the remaining VOCs onto the column. The analysis parameters, optimized as part of method development, are summarized in Table 2. GC-MS analysis was performed using an Agilent 5973N mass selective detector (Palo Alto, CA). Breath compounds were

identified and quantitated from resulting spectra using ChemStation® software (Agilent Technologies, Palo Alto, CA) and spectral database library from the US National Institute for Standards and Technology (NIST). Additional compound verification was provided using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) also available through NIST.

Table 2: SPME adsorption and sample analysis conditions

Fiber	65 µm PDMS/DVB
Extraction	30 min at 37°C
Desorption	5 min at 250°C
GC conditions	
Column	DB-WAXetr 30 m × 0.25 mm, 0.25 µm
Oven	50°C initial temperature 50°C to 100°C at 25°C/min to 150°C at 10°C/min (5 min hold) to 210°C at 10°C/min (5 min hold)
Carrier gas	2 ml/min (constant flow)
Injection port	Splitless, 0.75 mm liner, 250°C
MS conditions	
Mass range	34 to 350 (*m/z*)
Transfer line	250°C
Quadrapole	150°C
Scans/sec	4.5
Multiplier	2000 eV

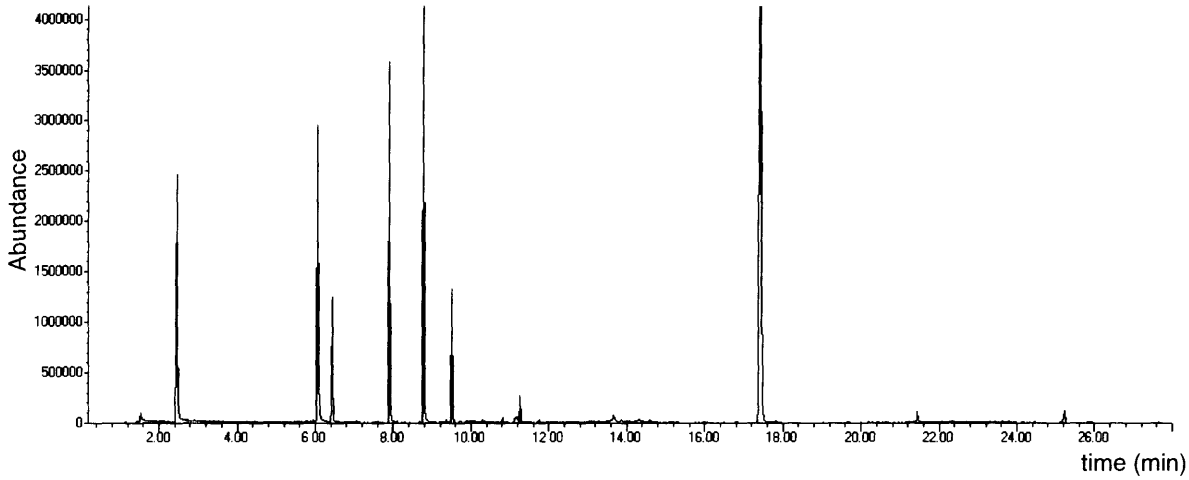

Figure 11: Typical chromatogram of breath

Compounds separated and analyzed by GC-MS produce peaks in the resulting total ion chromatogram (TIC) (Figure 11). A standard way of quantifying the amount of compound detected is to integrate the area under the curve, also referred to as area response (AR), of the most abundant ion in that peak. This measurement is related to concentration. Corrected area response (AR_C) refers to the area response of the MS for a particular compound relative to the area response for phenol. This normalization was needed to adjust for instrument fluctuations.

$$AR_C = \frac{AR_{VOC\ in\ breath}}{AR_{phenol}}$$

Many of the compounds found in breath are actually exogenous contaminants from the environment. Some of these exogenous compounds are also produced locally in the body at levels above or below the concentrations found in the environment. Blanks caused by sampling apparatus and ambient air were observed for comparison. A single SPME fiber was exposed for approximately 30 min to laboratory air, the same in which breath samples were collected. Concentrations of breath compounds in ambient air were compared to quantities found in breath. Sampling apparatus blanks were obtained by

filling collection bags with nitrogen gas and were analyzed with the same conditions as breath samples (Table 2). Sampling system blanks were used to evaluate the bag cleaning protocol and to identify sample contaminations arising from the sampling apparatus. VOCs were defined as breath compounds if they were found in the breath at significantly higher concentrations than in the background signal. However, a large group of compounds were found in breath at significantly lower concentrations than in the background. Kinetics analysis reveals that with these compounds the rate of clearance, by hepatic and/or renal pathways for example, is greater than the rate of introduction to the body from the environment. The concept of alveolar gradient was developed to distinguish between the two classes of breath chemicals. Although only compounds with positive alveolar gradients are produced endogenously, both positive and negative compounds can provide important physiological information [24]. Using AR, the alveolar gradient of each breath VOC was determined according to:

$$(AR_{VOC\ in\ breath} / AR_{phenol}) - (AR_{VOC\ in\ air} / AR_{phenol})$$

2.2 Method Development Experiments

2.2.1 Selection of SPME Coating

Solid-phase Microextraction (SPME) is a solvent-free concentration method for the extraction of VOCs from breath. Thin polymeric films which coat a fused-silica core, give the SPME fiber selectivity for certain types of chemicals. Analytes partition into the coating based on their physiochemical properties and sampling conditions. Choosing a proper SPME fiber coating is crucial to the effectiveness of sample analysis. Some useful and specific factors should be taken into consideration, such as polarity, molecular weight, sample matrix, concentration range, and sampling temperature.

Nonpolar coatings, e.g. polydimethylsiloxane (PDMS), are best used with low polarity hydrocarbons. Carboxen™ (CAR) and divynlbenzene (DVB) fibers work well for moderate to high concentration samples, and absorptive fibers are better for low concentration samples. Since breath compounds are expected in nanomolar concentrations, the following 4 fibers were chosen and compared:

85 µm StableFlex™ Carboxen™/Polydimethylsiloxane (CAR/PDMS)

65 µm Carbowax®/Divinylbenzene (CW/DVB)

65 µm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)

50 /30 µm StableFlex™ Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS)

A list of target breath chemicals was developed to simplify the task of quantification. Target chemicals were selected on the basis of frequency in breath samples. The area responses for target breath chemicals were monitored. Comparison and selection of fibers was based on extraction ability measured by both the number of compounds extracted and the quantitated area responses for each target analyte in the sample. PDMS/DVB, which is well suited for volatiles, amines, and nitroaromatic compounds, performed best and was used for all subsequent experiments. See section 3.2 for further details.

2.2.2 SPME Fiber Extraction

An important step in developing a SPME method for chemical analysis is determining the appropriate time needed for the analytes to reach equilibrium. Equilibrium is defined as the time above which additional fiber exposure to the analyte produces only minimal increase in concentration. It is determined by the analyte concentrations, the properties of the polymer coating, and sampling conditions. Adsorptive equilibrium is usually attained in 2 to 30 min. The extraction time profiles were established by plotting detector

response against extraction time (1, 10, 20, 30, 40, 120 min) for simultaneously collected breath samples.

2.2.3 Gas Chromatography-Mass Spectrometry Settings

Breath compounds were separated by gas chromatography and quantitated in a mass spectrometer (HP6890 and mass selective detector 5973, Hewlett-Packard, Palo Alto, CA). This split/splitless injector was operated at 250°C with a purge flow of 47.9 ml/min and 24:1 split ratio. Compounds were thermally desorbed in splitless mode for 5 min and afterwards the splitter was opened and the fiber removed. Injector port pressure was 16.75 PSI with carrier gas (helium) 52.7 ml/min. For separation, a DB-WAXetr capillary column, 30m length × 0.25mm i.d × 0.25µm film thickness, was used with 2.0ml/min column flow. Column temperatures were programmed as follows: 50° C for 5 min, 25° C/min to 100° C, 10° C/min to 150° C, 5 min hold, 10° C/min to 210° C, 5 min hold. MSD parameters were: ion mass/charge ration, 34 – 350 m/z; quadrupole temperature, 150°C; electron ionization temperature 230°C; interface temperature 250°C; electron multiplier voltage 1490 eV; scan resolution, 4.2 scans/s. See Table 2 for a summary of analysis conditions.

The best results for GC-MS pattern recognition are achieved when biomarker features are well resolved. It is believed that in this application features will correlate to compound peaks in the TIC. Therefore, the goal in GC temperature optimization was to achieve the greatest sensitivity and compound resolution in the shortest time possible. GC temperature parameters are often determined by trial and error. We used a standard approach that begins with a constant ramp rate of 10° C/min. Initial temperature adjustments, isothermal holds, slower ramping, and faster rate ramps were explored in

the development of the GC temperature profile. Additionally, noise levels in each resulting signal were tracked as another indication of performance.

2.2.4 Breath Sample Storage

For patient sampling in future clinical trials, it may not always be possible to analyze samples immediately after collection. Samples may need to be stored for up to 72 hours before analysis. Losses can occur due to adsorption or diffusion through bag walls. The effect of sample storage time (0.5, 6, 12, 24, 48, 72 hours) on the concentration of breath compounds in Tedlar® bags was investigated. The quantities of target VOCs as well as signal-to-noise measurements were monitored. Since exhaled breath composition can vary from collection to collection, breath samples were simultaneously taken from the same individual and stored and analyzed according to the same conditions (n = 6). Three repetitions were performed for each storage time investigated.

2.3 Breath Characterization Experiments

2.3.1 Single Individual Variability

It has been established that there can be variability in the composition and quantity of chemicals in breath for the same individual sampled at different times. Disease biomarkers are valuable in diagnosis only if they surpass thresholds of normal variability. In order to study the degree of variability in breath samples of a single individual, we examined changes in the frequency and quantity of target compounds in breath. Samples from the same individual were taken on 10 non-consecutive days at different times of the day and without control for dietary, exercise, or other parameters. First-order statistics were established for target analytes over the 10 samples.

In order to attain a quantitative measure of the degree of variability, we utilized principal component analysis (PCA), a commonly applied multivariate pattern recognition approach in chemical analysis [53]. As powerful as PCA is, it is often used an exploratory tool to visualize general relationships between data. Score plots of the first two principal components can be applied as a form of cluster analysis, where investigators try to determine if samples fall into well defined groups.

The first step in PCA is to determine the similarity between sample vectors. Data is processed to create similarity matrices, where a numerical score is calculated to indicate the similarity between each pair of samples. Because cluster analysis is not applicable to the small sample sizes collected in this study, we used the standard scoring metrics from PCA to assign a numerical description to the variability of breath samples in a single individual. The scoring metrics used were correlation coefficient, Euclidean distance, and Manhattan distance. Correlation coefficient is a similarity measure. Values are between 0 and 1 (-1 and 1 in some applications). The closer the score is to 1 the more correlated the samples are. Euclidean distance, the standard distance metrics used in PCA, is a dissimilarity measure. The computed values are not limited (always > 0), but higher Euclidian scores correspond to increasingly dissimilar samples. The Manhattan distance is very similar to the Euclidean distance; it is a dissimilarity score and by definition is always greater than the Euclidean distance.

Euclidean distance: $$D_{kl} = \sqrt{\sum_{j=1}^{J} (x_{kj} - x_{lj})^2}$$

Manhattan distance: $$D_{kl} = \sqrt{\sum_{j=1}^{J} \left| x_{kj} - x_{lj} \right|}$$

The distance metrics were applied to raw GC-MS total ion chromatograms (TIC), so each point was considered a unique feature. The distance or correlation to the average TIC was calculated for each observation. The mean distance was then reported as a measurement of the spread or variability between the sample observations.

2.3.2 Inter-individual Variability

Unrelated individuals were solicited to volunteer breath samples according to the collection protocol described above (n = 7 people). Collections were taken in the same laboratory environment to minimize variation differences in ambient air quality. While time of last meal, smoking habits, and medical conditions can greatly affect the VOC content in breath and the quality of samples for variability analysis, this study did not control for these parameters because practical applications will also not contain these controls. These factors, however, were recorded. The goal of this experiment is to identify normal breath variability across subjects irrespective of these factors. Results are meant to provide meaningful information about the data expected from our future clinical trials where parameters such as those listed above will not be controlled.

Both GC-MS and GC-DMS systems were used to study inter-individual variability. This also represented the first opportunity to evaluate the robustness of GC-DMS for breath analysis. Two identical PDMS/DVB coated SPME fibers were simultaneously exposed to each sample for extraction. Based on equilibrium calculations, simultaneous extraction leads to reduction in extracted compound concentrations. However, the relative abundances of compounds are maintained in both SPME fibers, theoretically preserving the ratio of complex biomarkers embedded in the spectral signals. This is in contrast to serial extractions where the second fiber may extract drastically different relative abundances as a consequence of the complexities in adsorption kinetics. Euclidean

similarity scores were computed for GC-MS and GC-DMS spectra and compared to similarity scores developed to describe variations in single individual sampling. Principal component analysis (PCA) was performed to provide visual representation of single individual and inter-individual variability.

2.3.3 GC-DMS Methodology

The GC-DMS breath analysis method was developed to mirror as closely as possible the method optimized on the GC-MS system. Breath sample collection and SPME adsorption conditions were identical to those described for the GC-MS system previously. Compounds were separated by gas chromatography (HP5890) using a DB-WAXetr capillary column, 5 m length × 0.25 mm i.d × 0.25 µm film thickness, with a 2.0 ml/min column flow. The microDMx from Sionex was used for sample detection. The split/splitless injector was also operated at 250°C with a purge flow of 47.9 ml/min and 24:1 split ratio. Column temperatures were programmed identically as they were for the GC-MS system described earlier. Refer to Section 2.3 and Table 2 for further information.

3 RESULTS

3.1 Preliminary Breath Experiments

These preliminary results are not the product of particular experiments for method development or signal characterization. However, they were a required to gain a basic understanding of the nature of breath samples collected with our protocol. This included an analysis of ambient air, elucidation of background due to the breath collection apparatus, as well as initial qualitative studies of breath samples. Preliminary experiments served several purposes: 1) to provide a means to verify that GC-MS results were consistent with those found in literature, 2) to determine if sample collection bags could be reused throughout the study, and 3) to identify signal background and determine its origin.

Signal analysis was performed with ChemStation® software and the automated mass spectral deconvolution and identification system (AMDIS). For an initial study of breath compounds and signal contamination from background, 15 samples from a single individual were taken on different days with variation in the time of collection. Ambient air (n=5) and sample collection apparatus blanks (n=3) were taken. Typical chromatograms of each are shown in Figure 12 with corresponding chemical identifications listed in Tables 3-5.

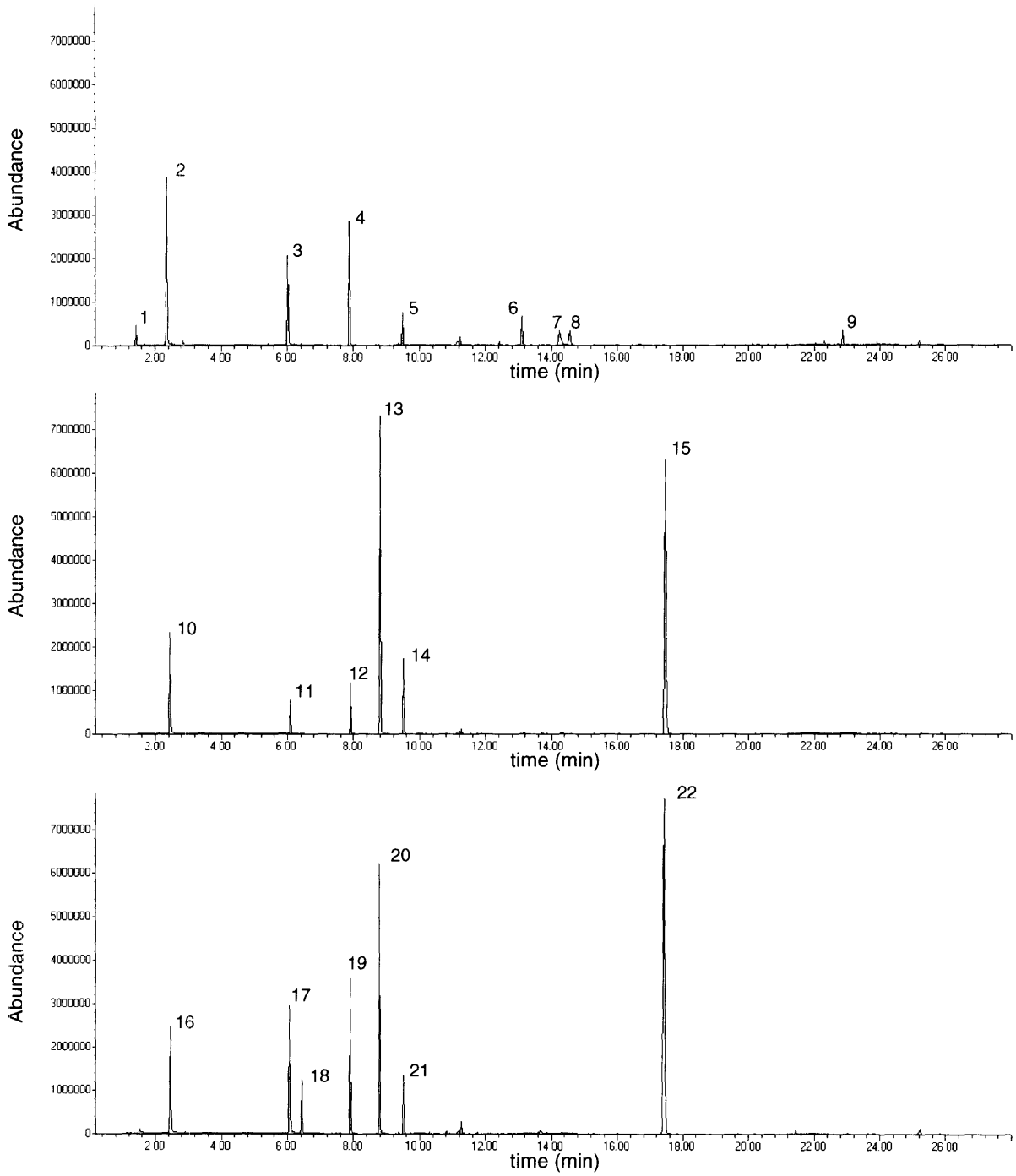

Figure 12: Representative chromatograms for ambient air (top), breath collection apparatus (middle), and breath sample (bottom) -- peaks identified in Tables 3, 4, and 5 respectively.

Table 3: Compound identifications from ambient air chromatogram, Figure 12 (top)

Compound	Peak no.	Retention time (min)	Match
Cyclotrisiloxane, hexamethyl-	1	1.42	96
Tetrachloroethylene	2	2.875	98
Cyclopentasiloxane, decamethyl-	3	6.025	93
Cyclohexasiloxane, dodecamethyl-	4	7.87	93
Cycloheptasiloxane, tetradecamethyl-	5	9.493	89
Ethanol, 2-(2-butoxyethoxy)-	6	13.099	91
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4 trimethylpentylester	7	14.232	85
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	8	14.552	85
Diethyl Phthalate	9	22.859	95

Table 4: Compound identifications for breath apparatus chromatogram, Figure 12 (middle)

Compound	Peak no.	Retention time (min)	Match
Cyclotetrasiloxane, octamethyl-	10	2.412	96
Cyclopentasiloxane, decamethyl-	11	6.025	74
Cyclohexasiloxane, dodecamethyl-	12	7.87	93
Acetamide, N,N-dimethyl-	13	8.769	96
Cycloheptasiloxane, tetradecamethyl-	14	9.493	90
Phenol	15	17.367	97

Table 5: Compound identifications for breath sample chromatogram, Figure 12 (bottom)

Compound	Peak no.	Retention time (min)	Match
Cyclotetrasiloxane, octamethyl-	16	2.454	95
Cyclopentasiloxane, decamethyl-	17	6.052	94
D-Limonene	18	6.433	94
Cyclohexasiloxane, dodecamethyl-	19	7.891	93
Acetamide, N,N-dimethyl-	20	8.786	94
Cycloheptasiloxane, tetradecamethyl-	21	9.507	90
Phenol	22	17.416	97

3.1.1 Identification of breath VOCs by GC-MS

Analysis using standard peak integration resulted in average detection on the order of 280 discernible peaks in the chromatograms of breath (n=56). Many of these peaks were not present in chromatograms of ambient air or the sample collection apparatus and therefore resulted from exhaled breath samples. Several breath compounds were found at low signal levels compared to compounds identified in Figure 12. Figure 13 is an expanded view of a chromatogram of breath and is shown to provide a better representation of breath compounds in the TIC.

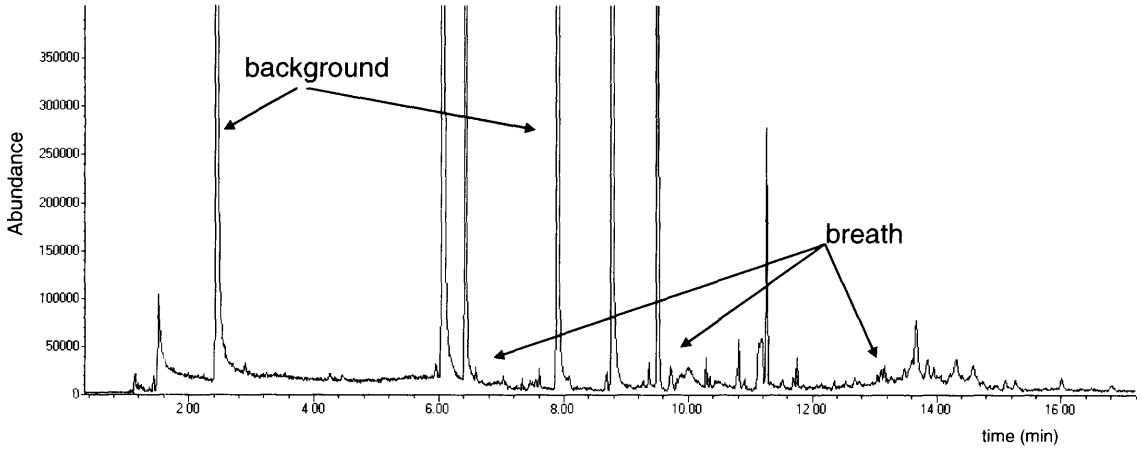

Figure 13: Representative chromatogram of breath.

Of the 15 breath samples studied of a single individual, 120 compounds were identified with various degrees of certainty (Table 6). "Identified" compounds are classified as those that met the requirement of having a spectral library match factor greater than 70 and were consistently found in at least 25% of samples. Match factor is described as the simple dot product between a library spectrum and the spectrum of the peak being identified [54]. A match factor of 100 represents a perfect match. Detected VOCs are mainly of 8 types, which are alkenes, alcohols, alkanes, aldehydes, ketones, volatile fatty acids, esters and other organic compounds. VOCs are grouped as either positive or

negative alveolar gradient. Section 2.1 provides a definition of these terms and a brief explanation of their physiologic significance.

Table 6: Identified Breath VOCs

Breath Compound	Retention time (min)	Match
----- *VOCs with positive alveolar gradients* -----		
Cyclopropane	1.044	89
Isoprene	1.143	77
Carbon disulfide	1.255	87
Dimethyl sulfide	1.303	87
Acetone	1.446	93
Dimethyl selenide	1.495	81
Octane, 2,2,6-trimethyl-	1.992	86
Ethanol	2.039	95
Sulfide, allyl methyl	2.235	73
3-Ethyl-3-methylheptane	2.38	81
1R-α-Pinene	2.878	85
Octane, 2,6-dimethyl-	3.057	85
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	4.447	85
3-Carene	5.533	92
β-Myrcene	5.953	90
DL-Limonene	6.421	94
Dodecane	6.436	76
Eucalyptol	6.501	88
Furan, 2-pentyl-	6.93	76
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	6.965	79
Benzenemethanol, α,4-dimethyl-	7.009	72
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	7.038	85
Styrene	7.229	84
Benzene, 1-methyl-2-(1-methylethyl)-	7.336	86
Benzene, 1-ethyl-4-methyl-	7.462	71
Octanal	7.552	81
Tridecane	7.604	89
Butane, 2,2-dimethyl-	8.204	70
Allyl Isothiocyanate	8.411	75
Tetradecane	8.674	85
Nonanal	8.687	76
Benzene, 1,4-dichloro-	9.302	80
Acetic acid	9.361	95
Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	9.443	73
Pentadecane	9.726	76
α-Cubebene	9.766	72
Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	9.777	74
Decanal	9.814	77
1H-Pyrrole, 1-butyl-	10.039	70
Propanoic acid	10.278	93
Propanoic acid, 2-methyl-	10.625	90
Caryophyllene	10.904	77
Butyrolactone	11.358	85
Bicyclo[4.2.0]octa-1,3,5-trien-7-ol	11.522	82
Heptadecane	11.89	75
Dodecanol	12.074	72
Naphthalene	12.529	82
Anisole, p-allyl-	13.648	89
Pentanoic acid	13.957	78
Naphthalene, 2-methyl-	14.093	82

Table 6 (continued): Identified Breath VOCs

Breath Compound	Retention time (min)	Match
Phenol, 2,4-bis(1,1-dimethylethyl)-	14.232	86
Hexanoic acid, 2-ethyl-	15.973	78
Benzothiazole	15.985	83
1,1'-Biphenyl, 3-methyl-	19.109	86
1,4-Benzenedicarboxylic acid, dimethyl ester	21.43	94
Benzoic Acid	23.728	93
Indole	23.766	90
----- *VOCs with negative alveolar gradients* -----		
Propene	1.054	86
Hexane, tetradecafluoro-	1.067	71
Pentane	1.135	74
Ethane, 1,1,2-trichloro-1,2,2-trifluoro-	1.137	85
Hexane, 2-methyl-	1.139	84
Hexane, 3-methyl-	1.153	85
Heptane	1.18	82
Cyanic acid, ethyl ester	1.254	79
Ethane, isocyanato-	1.254	79
Hexane, 2,4,4-trimethyl-	1.582	84
Heptane, 3,3,5-trimethyl-	1.671	85
Nonane	1.738	90
Pentane, 2,2-dimethyl-	1.757	90
Propane, 1-(methylthio)-	1.944	89
Isopropanol	1.975	93
Heptane, 2,2-dimethyl-	2.095	87
Pentane, 3-methyl-	2.144	78
Heptane, 2,2,4,6,6-pentamethyl-	2.498	88
Decane	2.6	86
1-Propanol	3.243	93
Undecane, 2,8-dimethyl-	3.402	84
Pentane, 3-methyl-	3.459	74
Undecane	4.25	80
p-Xylene	5.466	84
Camphene	6.553	81
Benzene, 1,2,3-trimethyl-	6.806	75
1,3-Cyclopentadiene, 1,2,3,4,5-pentamethyl-	7.458	78
Nonane, 2-methyl-5-propyl-	7.519	74
2-Butanone, 3-hydroxy-	7.545	85
2-Propanone, 1-hydroxy-	7.743	88
Tritriacontane, 15,19-dimethyl-	8.198	71
Benzofuran, 4,5,6,7-tetrahydro-3,6-dimethyl-	9.686	79
1-Hexanol, 2-ethyl-	9.715	95
Benzaldehyde	10.181	92
2-Nonenal	10.247	88
1-Butanol, 2-methyl-	10.438	79
1-Heptanol, 4-methyl-	10.438	82
1-Pentene, 3,4-dimethyl-	10.448	75
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, acetate, (1α,2α,5α)-	10.527	82
Dimethyl Sulfoxide	10.588	88
Hexadecane	10.817	79
Butanoic acid	11.267	71

Table 6 (continued): Identified Breath VOCs

Breath Compound	Retention time (min)	Match
Menthol	11.335	83
Butanoic acid, 4-hydroxy-	11.358	86
Ethanone, 2-(formyloxy)-1-phenyl-	11.552	91
3-Cyclohexene-1-methanol, α,α4-trimethyl-	11.934	77
Benzaldehyde, 2,4-dimethyl-	12.521	74
Cinnamaldehyde, (E)-	13.799	76
Phenol, 2,4,6-tris(1,1-dimethylethyl)-	14.667	78
Butylated Hydroxytoluene	15.084	73
1-Dodecanol	16.375/ 20.493	96
2-Pyrrolidinone	17.867	93
Isophthalaldehyde	19.76	78
Ethanol, 2-phenoxy-	20.015	70
1-Hexadecanol	20.495	85
5-Hydroxy-4-octanone	21.592	72
Butanoic acid, anhydride	21.637	70
Ethanone, 1-(5-methyl-1-phenyl-1H-pyrazol-4-yl)-	23.527	75
1,4-Benzenediamine, N-(4-methoxyphenyl)-	23.572	88
p-Isopropenylphenol	23.975	89
Benzophenone	24.152	92
Pentanoic acid	13.957	78
Naphthalene, 2-methyl-	14.093	82

3.1.2 Sampling Bag Reusability

Sampling bags were cleaned and reused in these experiments. Cleaning was achieved by heating the bag for 5 min at 37°C followed by purging with nitrogen gas for 5 min. These steps were repeated an additional time to guarantee complete removal of remaining contaminants and breath sample. Blank signals taken after bag cleaning were compared to the acquired signals from new bag blanks and breath samples. Typical chromatograms of the three signals are overlaid in Figure 14, and it appeared that compounds present in the breath signal are absent in the signals from new and cleaned bags. The figure is expanded to show the chromatograms between 6 and 10 min, and chemicals from the breath have been identified.

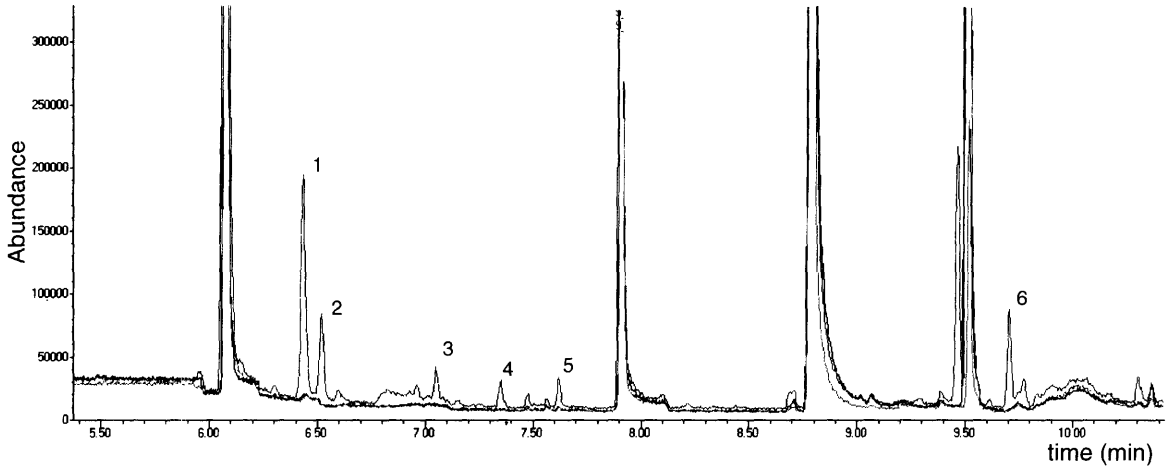

Figure 14: Overlayed chromatograms of breath sample (red), new bag (black), and cleaned bag (blue). Labeled chemical peaks are: 1) DL-Limonene, 2) Eucalyptol, 3) 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-, 4) Benzene, 1-ethyl- 2,4-dimethyl-, 5) Tridecane, 6) Benzofuran, 4,5,6,7-tetrahydro-3,6-dimethyl-

Quantitative analysis of the three samples revealed that 3.7% ± 1.3% of breath chemicals were found at reduced levels after the bag was cleaned. 25% indole, 11% Dodecane, and 1% DL-Limonene remained uncleaned from the bag. However, analysis of the new bag chromatograms showed that trace amounts of these uncleaned chemicals were also present, often at levels similar to or exceeding those found in cleaned bags. Additionally, new bags, which were purged but not heat treated prior to sampling, contained a greater number of breath chemicals. When levels in the new bags were accounted for, it was found that breath chemicals remaining after cleaning accounted for less than 0.1% of the signal seen in breath. The breath cleaning procedure was therefore found to be a sufficient requirement to permit bag reuse.

3.1.3 Identification of Background

As noted in Table 6, breath compounds are classified as either positive alveolar gradient or negative alveolar gradient. Alveolar gradient is a measure of the concentration of a compound in breath relative to the concentration in the ambient environment. The

significance of these gradients is discussed elsewhere in this thesis (Section 2.1). Several compounds were identified in the blank and ambient air chromatograms that were uniquely attributable to sources other than breath. These compounds were considered background and are listed in Table 7. Figure 12 (bottom) shows that these background compounds in the chromatogram significantly overwhelm breath compounds which can barely be seen at all. Furthermore, comparison of ambient air to background revealed three prominent compounds whose origins were determined to be from the bag material itself since no other differences between samples could account for their presence. Peaks corresponding to two of these compounds are labeled in the middle graphic of Figure 12: (13) Acetemide, N,N-dimethyl, (15) Phenol. The other is acetic acid.

Table 7: Identified VOCs in breath sample background

Background Compound	Retention time (min)
Carbon dioxide	1.04
Cyclotrisiloxane, hexamethyl-	1.492
Octane, 2,2,6-trimethyl-	1.977
Cyclotetrasiloxane, octamethyl-	2.412
Toluene	3.22
Cyclopentasiloxane, decamethyl-	6.025
1-Pentene, 4,4-dimethyl-1,3-diphenyl-1-(trimethylsilyloxy)-	6.507
Pentasiloxane, dodecamethyl-	7.384
Ethaneperoxoic acid, 1-cyano-1-phenylpentyl ester	7.459
Cyclohexasiloxane, dodecamethyl-	7.87
Acetamide, N,N-dimethyl-	8.769
Formamide, N,N-diethyl-	9.043
Cyclotrisiloxane, hexamethyl-	9.189
Cycloheptasiloxane, tetradecamethyl-	9.493
Benzaldehyde, 2,4-bis(trimethylsiloxy)-	9.991
2,3-Butanedione	10.346
Ethanol, 2-(2-methoxyethoxy)-	10.802
Ethanol, 2-(2-ethoxyethoxy)-	11.15
Benzene, propoxy-	11.19
Acetic acid, phenyl ester	11.209
Cyclooctasiloxane, hexadecamethyl-	11.247
Benzaldehyde, 3-ethyl-	11.485
Benzaldehyde, 4-methyl-	11.485
Acetophenone	11.519
2-Pyrrolidinone, 1-methyl-	11.685
Cyclohexane, isothiocyanato-	11.775
Acetic acid, phenylmethyl ester	12.38
Benzenemethanol, à-methyl-à-(1-methyl-2-propenyl)-	12.667
Diisopropylethylamine	12.797
Ethanol, 2-(2-butoxyethoxy)-	13.099
Cyclononasiloxane, octadecamethyl-	13.125
Butane,1,2,4-trichloro-heptafluoro-	13.648
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	14.552
N-Acetyl-S-(2-hydroxybutyl)-L-cysteine methyl ester	15.084
N-Formylmorpholine	15.303
Phenol	17.367
Quinoline, 1,2-dihydro-2,2,4-trimethyl-	19.655
Cyclooctasiloxane, hexadecamethyl-	20.137
Isopropyl Palmitate	21.336
Cyclononasiloxane, octadecamethyl-	22.032
Pentanedioic acid, (2,4-di-t-butylphenyl) mono-ester	22.306
Diethyl Phthalate	22.859
Cyclononasiloxane, octadecamethyl-	23.556
Benzene, 1,3,5-tri-tert-butyl-	23.825
Cyclononasiloxane, octadecamethyl-	25.708
Dibutyl phthalate	27.611

3.2 Method Development

3.2.1 Selection of SPME Coating

The sensitivity and selectivity of the four types of coatings to breath VOCs were evaluated. Each fiber extracted only a fraction of the 120 identified breath VOCs; average extraction (with percentage of total compounds) for each fiber is as follows: CAR/PDMS 29(24%), CW/DVB 28(23%), PDMS/DVB 38(32%), DVB/CAR/PDMS 43(36%). Table 8 summarizes the 22 breath target compounds extracted most frequently by all fibers and the frequency that each was detected.

Table 8: Most frequently extracted breath VOCs (n = 12 samples)

Target Breath Compound	Frequency
Acetone	100%
Limonene	100%
Propanoic acid	100%
Benzene, 1-methyl-2-(1-methylethyl)-	92%
Indole	92%
Sulfide, allyl methyl	83%
Pentanoic acid	75%
Propanoic acid, 2-methyl-	75%
Tetradecane	75%
Tridecane	75%
2-Pyrrolidinone	67%
Benzene, 1,4-dichloro-	67%
Benzenemethanol, α,4-dimethyl-	67%
Caryophyllene	67%
Undecane	67%
Ethanone, 2-(formyloxy)-1-phenyl-	58%
Pentadecane	58%
3-Carene	50%
Butylated Hydroxytoluene	50%
Dodecane	50%
β-Myrcene	50%
Dimethyl sulfide	50%

Triplicate samples were taken for each fiber. The 22 compounds were quantitated to asses the extraction efficiency of each fiber. For more meaningful comparison, the corrected area response of each analyte was normalized on a scale from 0 to 100 and plotted in groups for each target VOC (Figure 15). The SPME coating that achieved the

largest extraction for a target compound received a score of 100. The remaining fibers were assigned scores representing the percent of analyte extracted relative to the largest extraction. PDMS/DVB had the highest extraction capability in nine of the compounds. CW/DVB was superior to the other coatings for the extraction of only one compound, indole. CAR/PDMS and DVB/CAR/PDMS were each found to be most efficient at extracting six of 22 tested breath compounds.

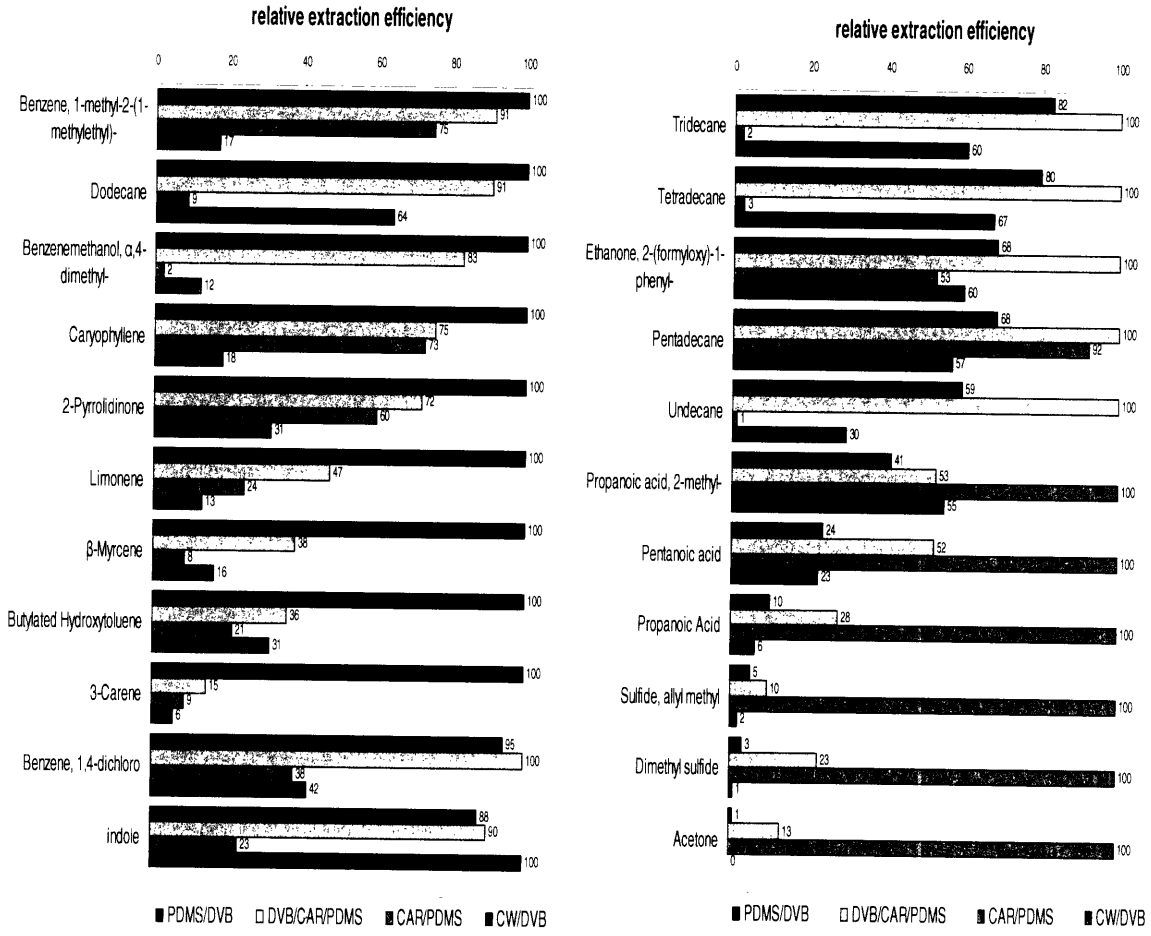

Figure 15: Relative extraction efficiency of SPME coatings to breath VOCs

3.2.2 SPME Extraction Conditions

Equilibrium is a condition when the concentrations of compound in the collection bag and on the SPME fiber do not change. The objective of SPME extraction is to reach distribution equilibrium in the system. However, in complex samples such as breath, what is found is that analytes compete for active sites on the solid porous polymer phase the divynlbenzene in PDMS/DVB fibers. Therefore, at longer extraction durations compounds in the breath matrix are displaced by other compounds for which the fiber has greater affinity. The optimal time for extraction time may not be the equilibration time if it is desirable to limit the effects of competitive displacement.

In order to determine the optimal duration for SPME sampling, extraction was monitored by plotting area response against fiber exposure time. Extraction profiles were established for the 22 most frequently detected chemicals, determined in Section 3.2 (Table 8). Compounds can be classified into four groups based on their observed extraction characteristics to prolonged extraction duration: increasing with equilibrium (IE), increasing with no equilibrium (INE), decreasing with equilibrium (DE), and decreasing with no equilibrium (DNE). The 22 compounds monitored were distributed across all extraction characteristics except for DNE. Representative plots of extraction profiles for each class are given in Figure 16: propanoic acid for IE, undecane for INE, and acetone for DE. An extraction time of 30 minutes was chosen for subsequent experiments because it seemed to best balance the competitive adsorptions of VOCs in breath. At this duration, extracted acetone decreased to 52% of its maximum, propanoic acid reached 92% of its equilibrium value, and undecane increased to 12 times its value initially extracted at 1 min (Figure 16).

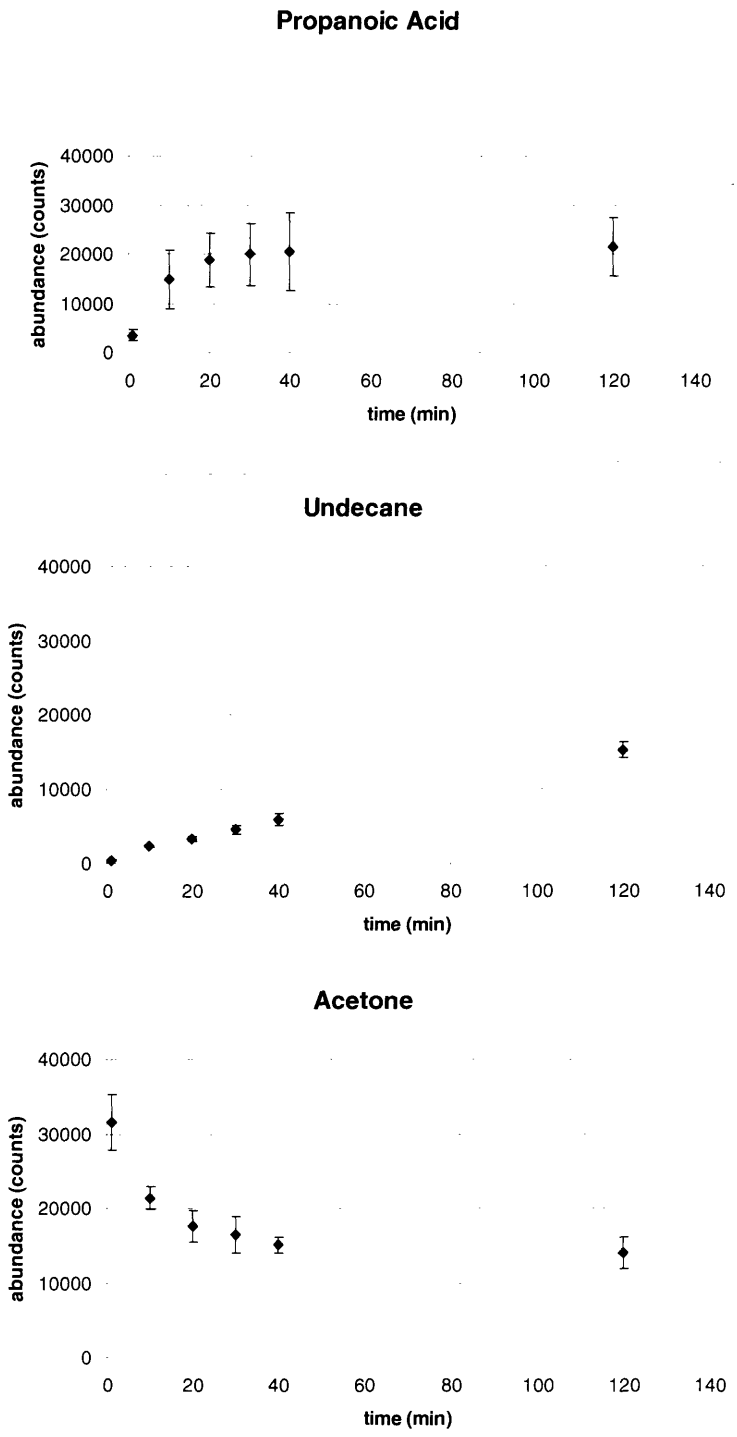

Figure 16: Representative extraction profiles for three classes observed. Values are given as the average (n = 3) ± standard deviation. IE (top), INE (middle), DE (bottom)

3.2.3 GC-MS Settings

In order to develop a gas chromatography temperature profile, performance was evaluated against three parameters:

1. Breath compound peak resolution
2. Overall signal-to-noise ratio
3. Analysis runtime

The left-hand panels in Figure 17 show the oven profiles that were developed and compared to optimize the stated goals. Although the retention time indexes are not constant due to different temperature profiles, the region between 6 min and 18 min in the resulting plots was targeted for improvements in component resolution. The sequence of TICs as subsequent methods showed marked improvement in signal separation. The signal noise levels were also monitored. A higher signal noise is associated with a higher noise level value. The final method chosen was method 5. While it did not achieve superior results in all performance measures, this was the method that provided the optimal balance between noise, run time, and peak resolution. Compounds were the most separated with this method and a noise level of 19 was the second lowest observed. Method 1 with a noise level of 18 had a run time of 40 min, which was less desirable than 28 min for sample analysis achieved with method 5.

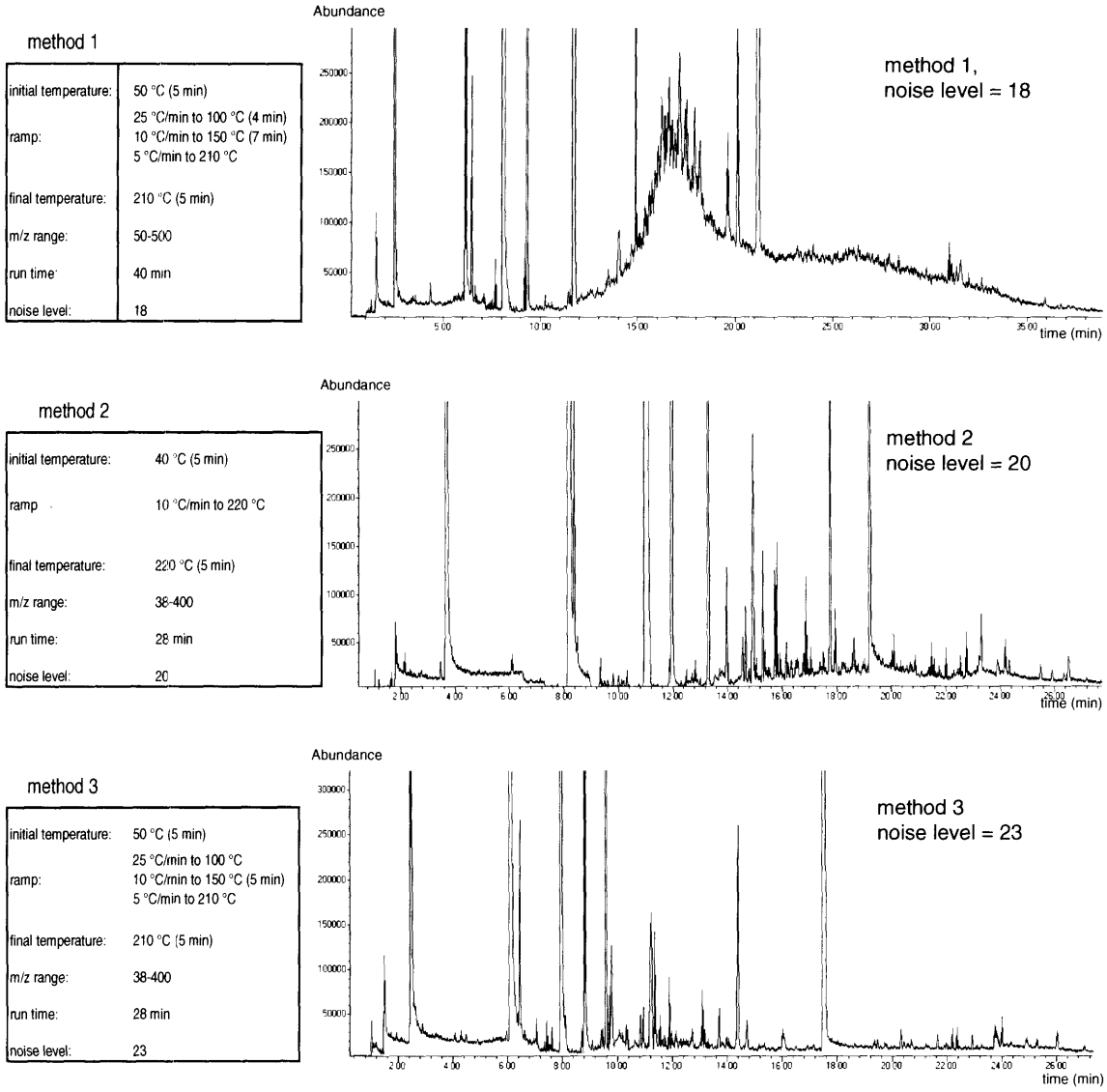

Figure 17: GC temperature profiles and the resulting total ion chromatograms (TICs). Adjustments to the oven temperature programs result in improved compound resolution.

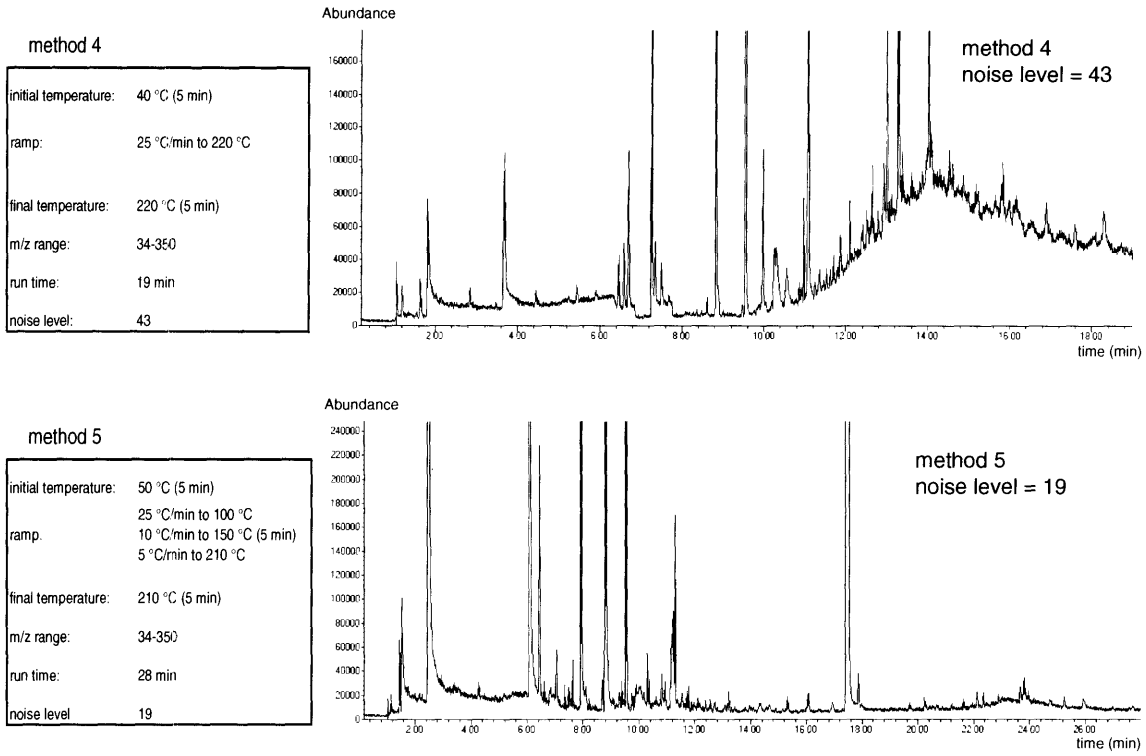

Figure 17 (cont): GC temperature profiles and the resulting total ion chromatograms (TICs).

3.2.4 Sample Storage Time

The results of sample stability at different storage durations did not reveal significant changes in the observed response to breath VOCs. When 72 hour holding was compared with 30 minute holding, observed breath sample responses for well known VOCs including acetone, propanoic acid, undecane, tridecane, β-Myrcene, ethanone, 2-(formyloxy)-1-phenyl-, and indole changed an average of 42.5%. Storage for 24 hours resulted in an average 29.8% change from baseline. These results appear to indicate that these compounds should not be stored for long periods of time (Figure 18). However, the above results were statistically qualified using a Student t-test with a 95% confidence. Comparisons were made between the mean values of samples analyzed after 30 min (n = 3) to the mean values of stored samples at (6, 12, 24, 48, 72 hrs). Although the average trends are suggestive, the results were not statistically significant

(Student t-test, $\alpha = 0.05$). The only VOC that achieved significance was tridecane. The 37.6% decline in response from 30 min to 72 hrs was statistically significant with $p = 0.029$.

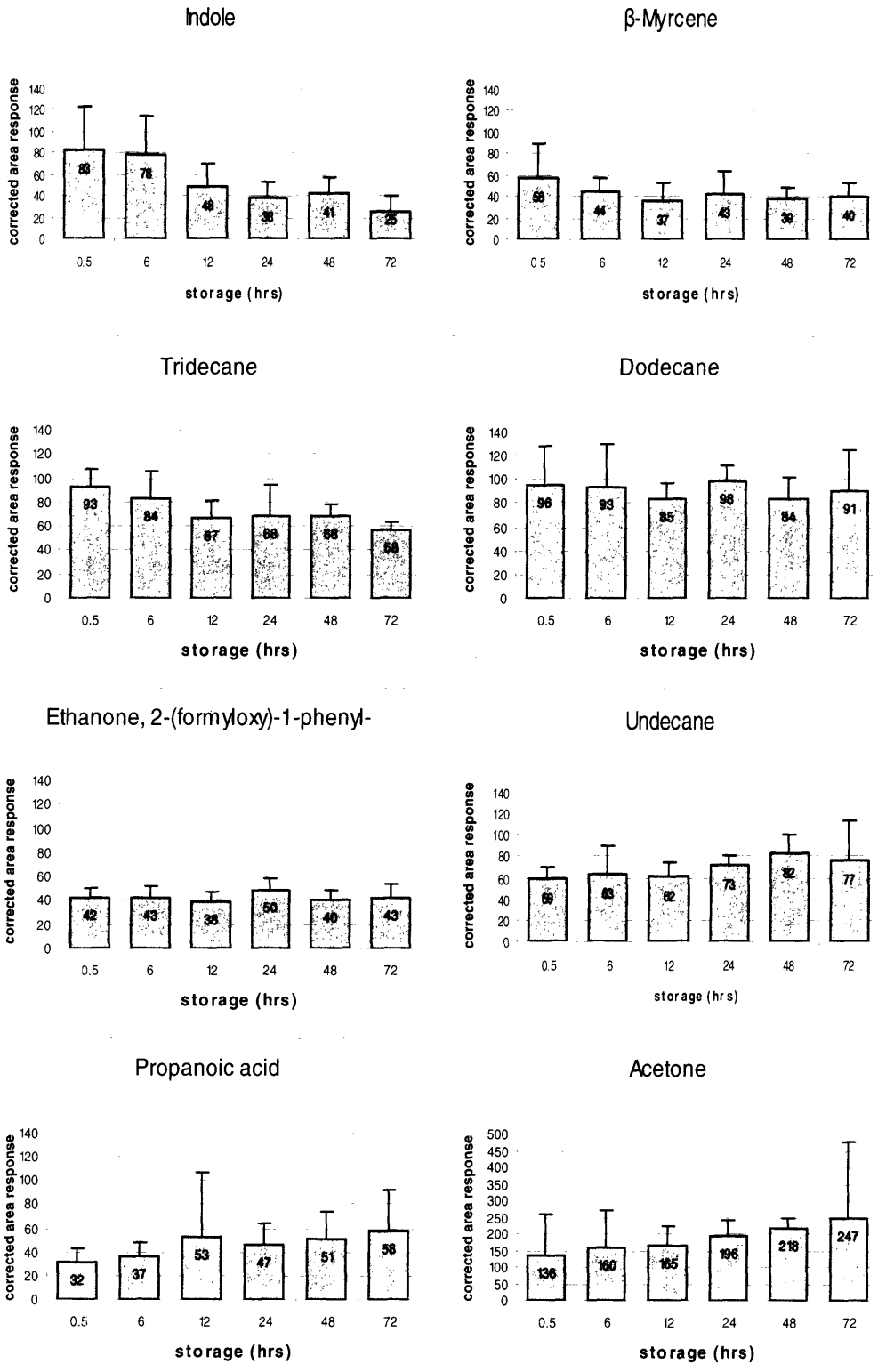

Figure 18: Storage stability of breath VOCs. Results are plotted as the average + the standard deviation

3.3 Breath Characterization

3.3.1 Single Individual Variability using GC-MS

In order to understand the possible variability in exhaled breath, the target VOCs that were observed with the greatest frequency (section 3.2.1) were analyzed in ten samples from a single individual. The frequency of breath compounds in these samples is summarized in Table 9. Although the sources of variability were not determined, variability from adsorption to sample collections bags, differences in SPME fiber extraction conditions, the time of day samples were taken, and even stress levels could all be contributing factors.

Table 9: Frequency of target breath VOCs in single individual (n = 10 samples)

Target Breath Compound	Frequency
Benzene, 1-methyl-2-(1-methylethyl)-	100%
Benzenemethanol, α,4-dimethyl-	100%
Limonene	100%
Tridecane	100%
Acetone	90%
Butanoic acid	90%
Indole	90%
Dodecane	80%
Tetradecane	80%
Ethanone, 2-(formyloxy)-1-phenyl-	70%
β-Myrcene	70%
2-Pyrrolidinone	60%
Propanoic acid, 2-methyl-	60%
Undecane	50%
Benzene, 1,4-dichloro-	40%
Butylated Hydroxytoluene	40%
Sulfide, allyl methyl	40%
3-Carene	20%
Caryophyllene	20%
Propanoic acid	20%
Dimethyl sulfide	0%
Pentadecane	0%

Corrected area responses were used to monitor variations in exhaled quantities of these compounds. Table 10 provides the mean corrected area responses for each with standard deviation and % deviation. The % deviation is provided as a means to compare the variability in different compounds. VOCs with higher % deviation have a greater degree of variability. Some of the compounds exhibited high degrees of variability, with % deviation ≥ 100%: Caryophyllene (117.3%), DL-Limonene (102%), and 3-Carene (100%). However, only DL-Limonene should be noted for this observation. The others were not observed with enough frequency to be significant. The results for Limonene, however, are significant since it was found in all breath samples analyzed.

Table 10: Target breath VOC variability in single individual (n = 10 samples)

	corrected area response		
Target Breath Compound	mean	standard deviation	% deviation
Benzene, 1-methyl-2-(1-methylethyl)-	13045.7	10010.7	76.7%
Benzenemethanol, α,4-dimethyl-	7571.1	5120.0	67.6%
Limonene	92090.6	93937.9	102.0%
Tridecane	8395.5	3415.2	40.7%
Acetone	22054.8	13650.0	61.9%
Butanoic acid	6881.0	1800.3	26.2%
Indole	7093.2	2683.2	37.8%
Dodecane	6042.5	2963.2	49.0%
Tetradecane	7036.9	5697.1	81.0%
Ethanone, 2-(formyloxy)-1-phenyl-	3334.1	1250.1	37.5%
β-Myrcene	5596.9	3378.2	60.4%
2-Pyrrolidinone	10804.8	2756.9	25.5%
Propanoic acid, 2-methyl-	1989.5	826.0	41.5%
Undecane	3374.0	795.2	23.6%
Benzene, 1,4-dichloro-	2285.0	857.4	37.5%
Butylated Hydroxytoluene	3347.6	2942.2	87.9%
Sulfide, allyl methyl	4690.0	3240.5	69.1%
3-Carene	27423.0	27418.8	100.0%
Caryophyllene	5039.1	5913.0	117.3%
Propanoic acid	6984.1	3315.3	47.5%
Dimethyl sulfide	0.0	0.0	0.0%
Pentadecane	0.0	0.0	0.0%

Correlation, Euclidean distance, and Manhattan distance provided a numerical measure of the similarity between the ten breath signals. For comparison, the same distance

calculations were applied to ambient air and background samples. Table 11 reports the distance from the sample average for each group. It is clear that breath samples have more variability than ambient air. However, the scores for breath are not statistically different from those for background using these scoring methods (pairwise Student t-test, $\alpha = 0.05$).

Table 11: Distance calculations for breath, air and background signals.

	Euclidean score			Manhattan score			Correlation score		
sample	breath	air	background	breath	air	background	breath	air	background
1	2.932E+07	1.277E+06	2.278E+07	7.027E+08	2.048E+07	2.543E+08	0.37726	0.00517	0.44044
2	3.501E+07	2.063E+06	1.179E+07	4.944E+08	1.825E+07	1.385E+08	0.32015	0.00855	0.10626
3	3.193E+07	2.508E+06	1.213E+07	4.388E+08	2.041E+07	1.321E+08	0.24909	0.00623	0.12265
4	3.094E+07			4.807E+08			0.23915		
5	2.287E+07			3.632E+08			0.30016		
6	2.346E+07			3.410E+08			0.30439		
7	2.784E+07			3.721E+08			0.34805		
8	4.378E+07			6.118E+08			0.36984		
9	2.986E+07			4.494E+08			0.33276		
10	5.633E+07			6.518E+08			0.42015		
average	**3.313E+07**	**1.949E+06**	**1.557E+07**	**4.906E+08**	**1.971E+07**	**1.750E+08**	**0.32610**	**0.00665**	**0.22312**

Exploratory principal component analysis (PCA) was also performed to provide a representation of the variability in the three data sets (Figure 19). From the PCA results we can observe that ambient air is tightly clustered together while background and breath samples are more spread out.

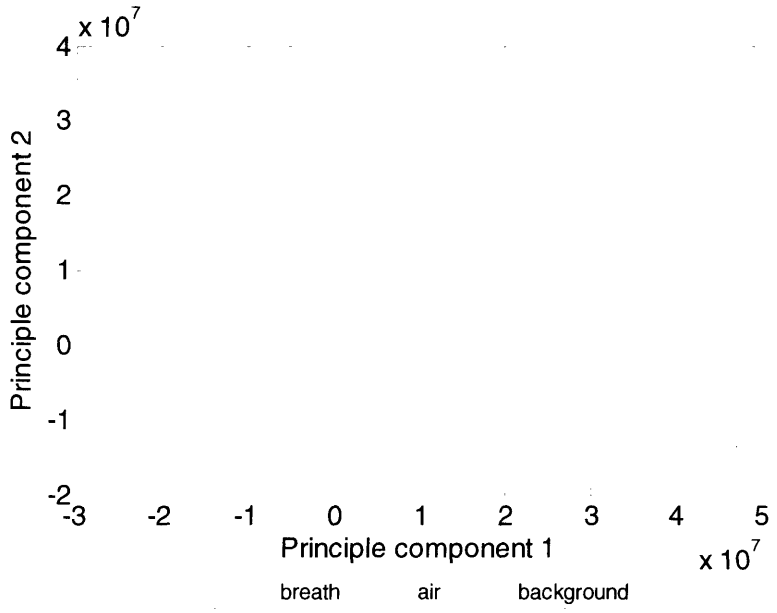

Figure 19: Plot of PCA scores for breath from a single individual, ambient air, and collection apparatus background

3.3.2 Inter-individual Variability

When more than a single individual is considered, the number of breath compounds identified can reach over three thousand [24]. The final experiment of this thesis aimed to look at the variability in breath from multiple subjects using our unique sampling methodology. Rather than providing a qualitative listing of compounds, a quantitative measurement of signal similarity was calculated. Correlation, Euclidean and Manhattan distances applied previously to single individual samples were used as the numerical metrics. PCA was used here to identify and visualize any trends in the data.

For this study, 7 individuals were solicited for breath samples. The sample selected was too small to represent the adult population, but represents one of the first SPME-GC-DMS analyses done on control human subjects. The mean age was 25 years old and there were 2 females and 5 males. All of the subjects were apparently healthy and 2 of 7 were known smokers. Samples were collected using collection methods and extracted with SPME (PDMS/DVB) as outlined above followed by analysis on GC-MS and GC-DMS systems according to the following parameters. Plots are shown in figures 21 through 25 and numerical results are reported in tables 12 through 15.

3.3.2.1 GC-MS Total Ion Chromatogram Variability

Euclidean, Manhattan, and correlation metrics were applied to total ion chromatograms (TICs). Less variation is observed across multiple subjects than within a single individual (Student t-test, $p = 0.0078$). Unique peaks were observed between individuals, but variability was not tremendous under the sampling and analysis conditions.

Table 12: Distance scores for single and multiple subject studies

	Euclidean score		Manhattan score		Correlation score	
sample	single subject	multiple subjects	single subject	multiple subjects	single subject	multiple subjects
1	2.932E+07	1.739E+07	7.027E+08	2.259E+08	0.37726	0.012
2	3.501E+07	3.599E+07	4.944E+08	2.490E+08	0.32015	0.059
3	3.193E+07	1.230E+07	4.388E+08	1.643E+08	0.24909	0.004
4	3.094E+07	1.077E+07	4.807E+08	2.308E+08	0.23915	0.005
5	2.287E+07	1.156E+07	3.632E+08	2.298E+08	0.30016	0.006
6	2.346E+07	1.577E+07	3.410E+08	2.123E+08	0.30439	0.008
7	2.784E+07		3.721E+08		0.34805	
8	4.378E+07		6.118E+08		0.36984	
9	2.986E+07		4.494E+08		0.33276	
10	5.633E+07		6.518E+08		0.42015	
average	**3.313E+07**	**1.730E+07**	**4.906E+08**	**2.187E+08**	**0.32610**	**0.01579**

Principle component analysis supports this finding (Figure 20). The TICs from multiple subjects were more tightly clustered than were the corresponding measurements for a single individual. Also, a peculiar finding was that ambient air, breath and background were all clustered away from the multiple subjects. This unexpected outcome suggests that some experimental bias has affected these results.

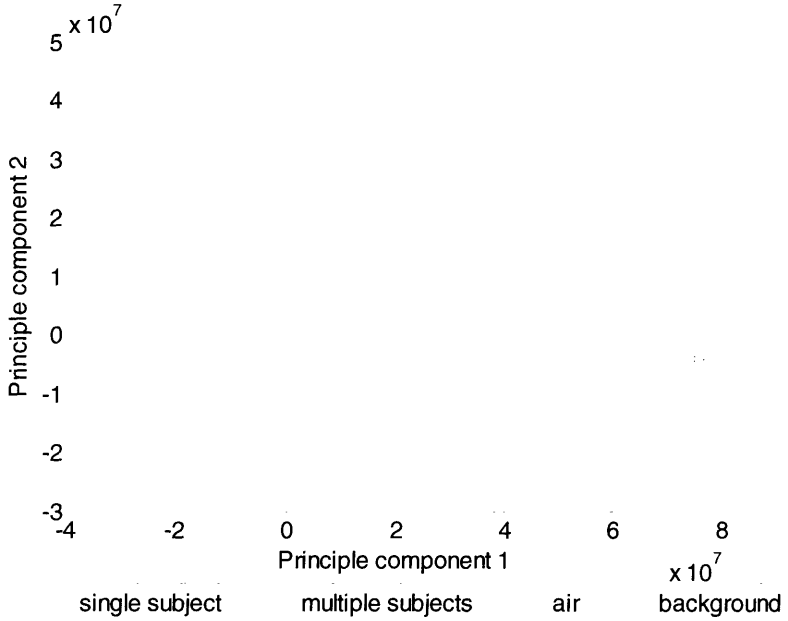

Figure 20: Plot of PCA scores for breath from a single individual, breath from multiple subjects, ambient air, and collection apparatus background

Figure 21 shows representative chromatograms from the single and multiple subject studies. Although TICs from section 3.1 suggest the largest peaks result from background chemicals, the two representative TICs here do not have backgrounds that match. Moreover, the abundance of peaks for phenol and acetemide, N,N-dimethyl- are noticeably greater in the typical chromatogram of a subject in the inter-individual variability study. At the beginning of this study, a new set of sample collection bags were ordered. It appears from this figure that the release of tedlar VOCs is more substantial in the new set as more prominent peaks are produced. This confounding finding makes comparison between the two data sets more difficult.

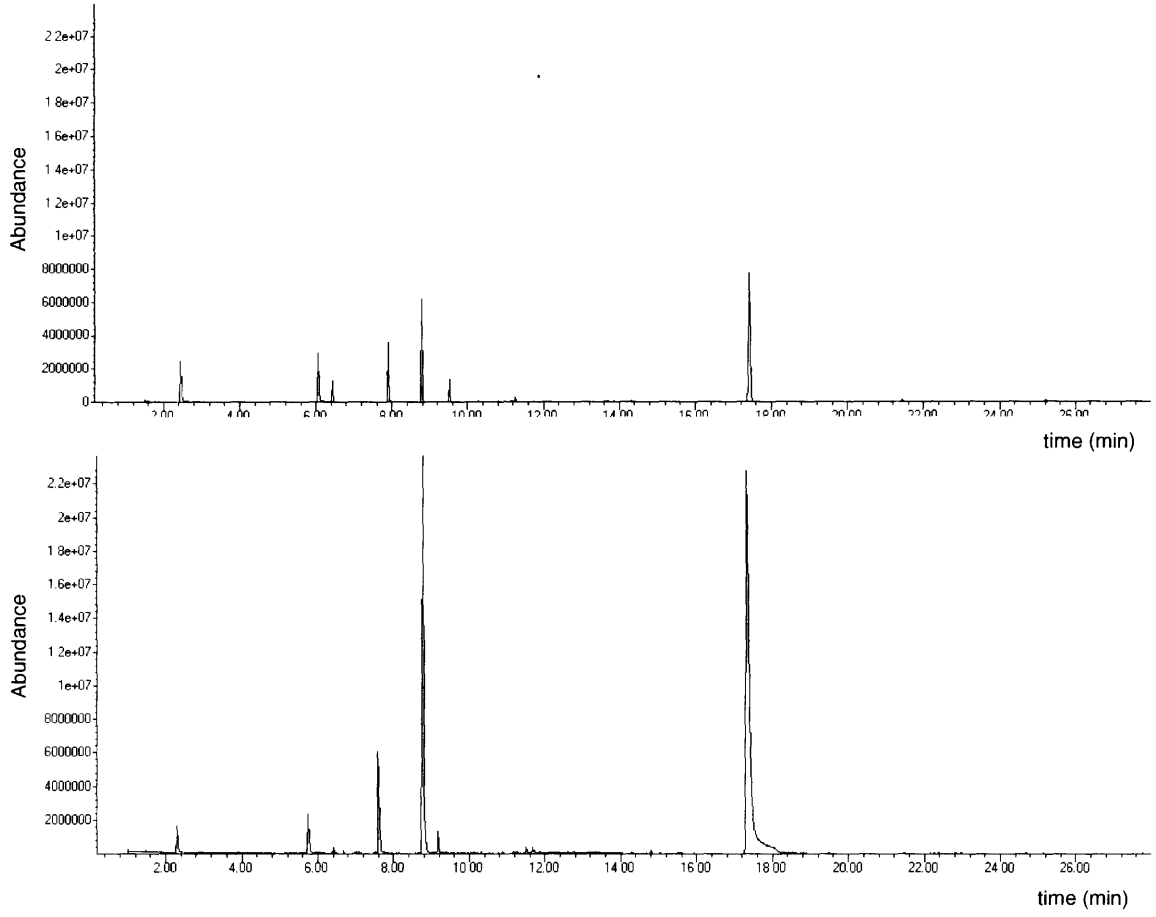

Figure 21: Typical chromatograms of breath from signal individual (top) and typical breath from one of the multiple individuals (bottom).

3.3.2.2 GC-DMS Feasibility and Total Ion Abundance Variability

As this is one of the first examples of GC-DMS analysis of breath it will be important to show that there is not only consistency between sample but that also the expected variation is also present. Representative DMS spectra are shown in Figure 22. Breath from two representative subjects are compared at 3 different times during the run of our standard method. The plots illustrate how there are many similarities between the acquired signals but also illustrate that the differences between individuals can be appreciated qualitatively.

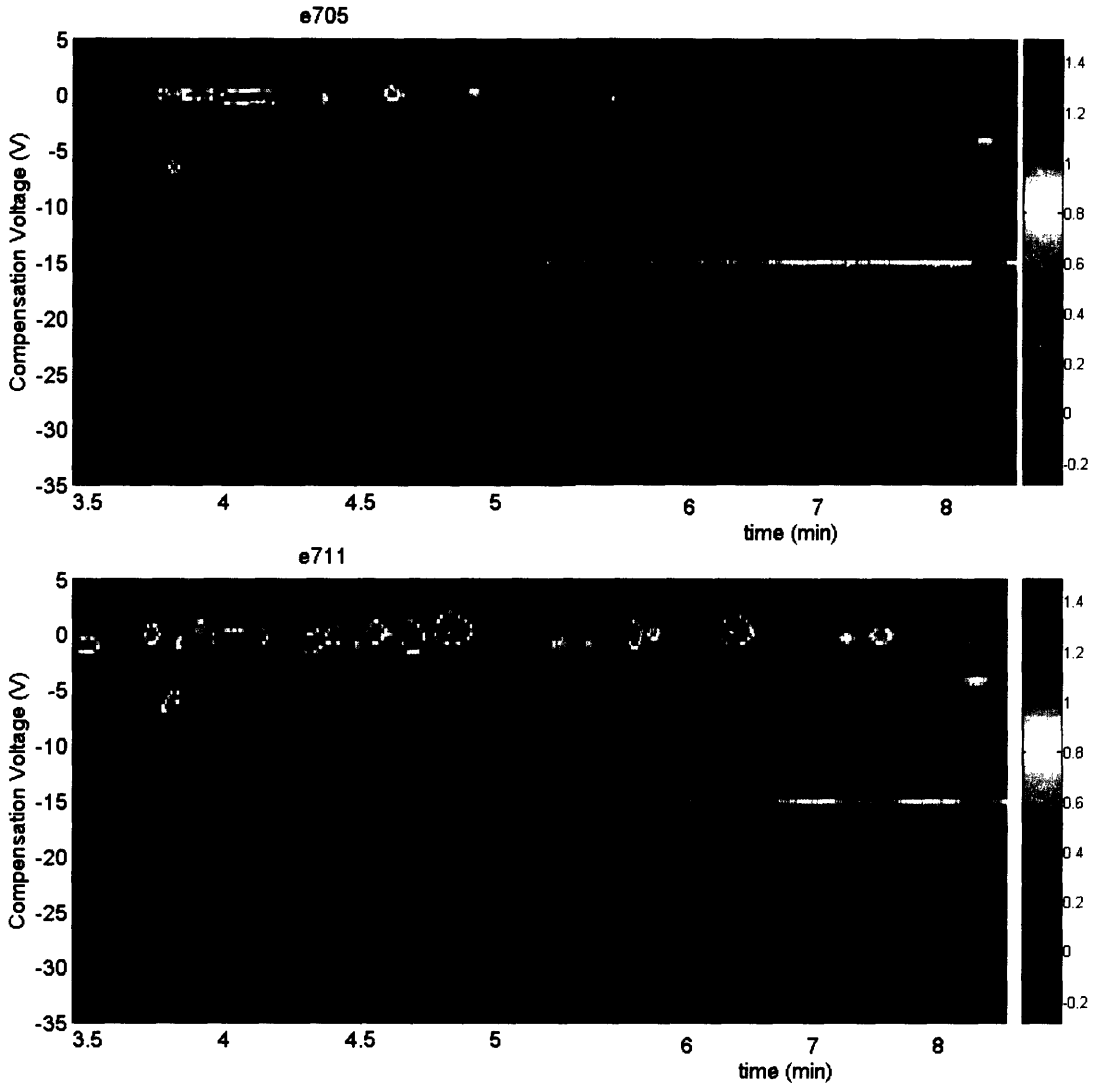

Figure 22: Representative SPME-GC-DMS spectra from 2 subjects highlighting the similarities and differences between acquired spectra. Subject 705 (top) and subject 711 (bottom). Colors represent signal intensity at the corresponding time and compensation voltage, and scaling is the identical for each plot.

In order to perform numerical analysis, a signal representation analogous to total ion chromatogram (TIC) was created. Total ion abundance (TIA) is the result of summing intensities across all compensation voltages in the positive ion DMS spectrum. Two representative TIAs are shown in Figure 23.

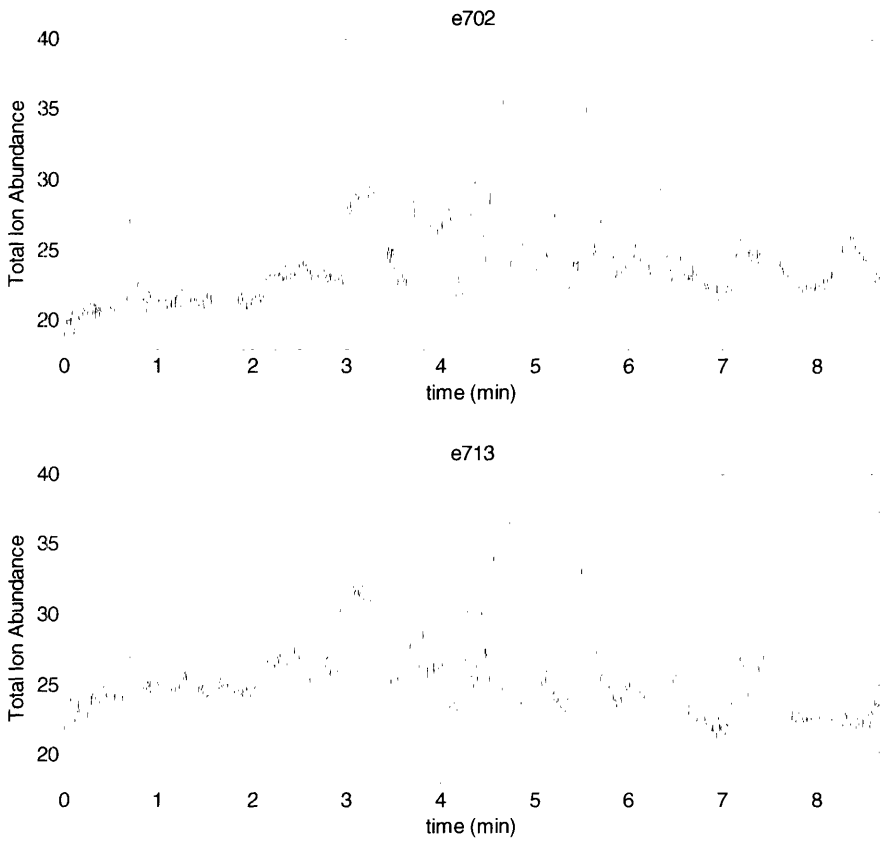

Figure 23: Total Ion Abundance signals from SPME-GC-DMS analysis of breath from two subjects.

Distance measurements were performed resulting in the following matrices. Here the entire matrices are given for each distance function to illustrate how some breath TIAs are more related than others. Each subject pair produces a single score. Therefore, only scores in the lower triangle need to be observed. The distance to the sample average is also provided in the right most column of the matrix.

Table 13: Euclidean distance measurements of breath TIAs

subject number	e701	e702	e703	e704	e705	e711	e713	distance to average
e701	0							93.73
e702	120.28	0						41.73
e703	134.06	44.82	0					59.24
e704	130.03	77.65	87.57	0				56.33
e705	131.82	65.76	81.39	58.36	0			52.95
e711	91.82	52.19	61.14	86.25	76.83	0		38.23
e713	107.97	89.16	108.95	77.91	85.47	85.50	0	61.94

Table 14: Manhattan distance measurements of breath TIAs

subject number	e701	e702	e703	e704	e705	e711	e713	distance to average
e701	0							1627.30
e702	2346.70	0						923.55
e703	2906.70	1029.70	0					1470.20
e704	2005.90	1775.80	1990.80	0				1224.50
e705	2211.60	1437.40	1988.50	1206.60	0			1008.50
e711	1878.60	988.16	1513.60	1938.20	1438.80	0		869.02
e713	1720.10	1983.50	2548.10	1451.90	1727.50	1991.40	0	1337.80

Table 15: Correlation coefficients for breath TIAs

subject number	e701	e702	e703	e704	e705	e711	e713	correlation with average
e701	0							0.202
e702	0.428	0						0.132
e703	0.356	0.101	0					0.089
e704	0.690	0.586	0.542	0				0.376
e705	0.831	0.503	0.517	0.492	0			0.463
e711	0.258	0.158	0.066	0.612	0.551	0		0.084
e713	0.501	0.496	0.506	0.457	0.731	0.505	0	0.290

These distance functions show that there was inter-sample variability (Table 13-15). Principle component analysis verifies this and one observation is found in the left half of the plot (Figure 24). This outlier is subject e701, the same subject that had the largest Euclidean and Manhattan distances when compared to other subjects. The TIA for this sample is shown in Figure 25.

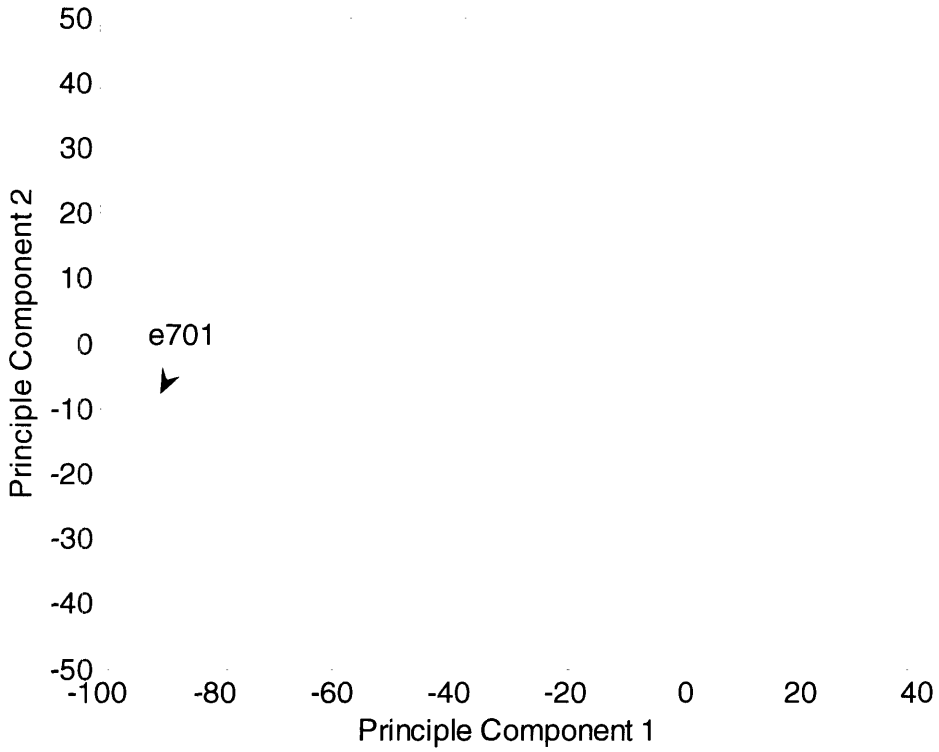

Figure 24: Plot of PCA scores for breath from multiple individuals.

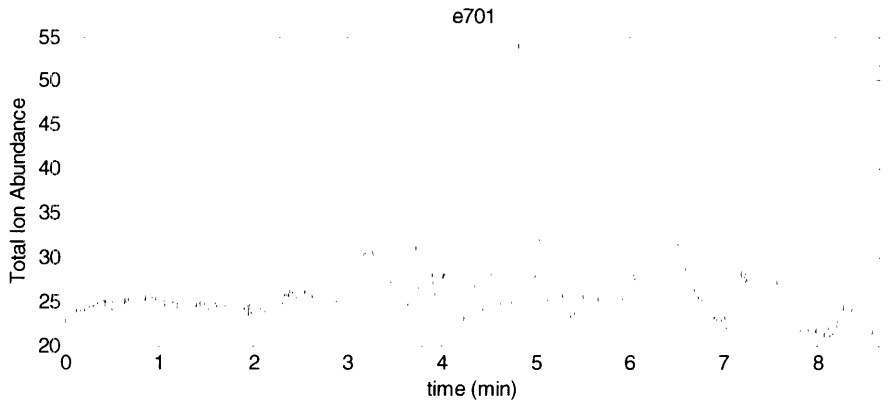

Figure 25: Total ion abundance of breath sample from subject e701

4 DISCUSSION

4.1 Preliminary Breath Experiments

The purpose of these preliminary experiments was to establish that our GC-MS method could be used to identify breath compounds, to ensure that our protocol supported the reuse of sampling bags, and to identify components of the breath sample background. Discussion is also provided here regarding the collection method and materials used in this thesis.

4.1.1 Breath Collection Method

One of primary advantages of breath analysis is that it has the potential to be a more convenient and non-invasive way to obtain medically relevant measurements. As simple as it is to breath, obtaining proper samples of breath for analysis is still a challenge. There are currently no accepted methodologies to collect breath for analysis. And one limiting feature is that there are many opportunities for contamination from the environment, from the collection apparatus, and even from airways inside the body. Some researchers employ lung washouts with pure air [9, 34], some require subjects to breath into complex instruments [31, 55], and others suggest subjects to hold their breath for between 20 and 60 seconds before giving a sample [51]. While accuracy is sought, so are convenience and a method that would be practical in the clinical setting where breath analysis will be carried out. The collection protocol established in this research aimed to balance accuracy with convenience. In order to increase accuracy, once the method had been developed, it did not change during the study although new information (e.g. reduction of background with bag purging) provided evidence that it could be modified to improve results.

4.1.2 Identification of breath VOCs by GC-MS

In preliminary studies, 120 compounds were identified in breath although many more compounds were found that did not pass identification criteria. In other investigations where the primary objective was to establish the full range of compounds in normal human breath, over 3000 VOCs were observed [24]. Possible reasons for the discrepancies between results are: 1) breath samples were directly concentrated using sorbent tubes before analysis with GC-MS as opposed to SPME extraction from sampling vessels, 2) the collection and analysis methods employed permitted a greater number of compounds to surpass the threshold for identification, and 3) the study analyzed breath samples from 50 individuals while only a single subject was used for compound identification in this thesis. However, the study reported mean detection of 204 compounds for single individuals – a number more consistent with the results observed here.

Additional similarities exist between VOCs identified in previous studies and the present work. Most notably, the majority of the 120 compounds identified here were found among the most frequently observed constituents of breath [24, 31]. Determination of positive and negative alveolar gradient VOCs was also congruent to previous results. This work represents one of the few elaborations of breath VOCs using solid phase microextraction and the observed similarities to past results serve to validate the experimental parameters determined in this thesis.

One of the difficulties in compound identification was achieving match factors above our threshold of 70. Peak spectra can be "contaminated" with extraneous ion masses which can arise from co-eluting compounds, column bleed, and ion-chamber contaminants [54]. These masses can make it difficult to perform automated compound identification.

This work represented the application of AMDIS for compound identification in addition to more traditional methods used in Chemstation software. AMDIS, developed in 1998, is a technique that extracts "purified" spectra from peaks in the total ion chromatogram (TIC) for comparison with reference libraries [56]. This permits reliable detection performance at very low signal levels and for overlapping compounds in the TIC. These features were particularly useful as many extracted breath compounds provided only low signal amplitudes. Moreover, AMDIS aided in the identification of coeluting breath compounds and helped expose regions of the TIC that required greater resolution.

4.1.3 Breath Collection Materials

In most studies, breath was collected in inert tedlar bags or stainless steel electropolished canisters [3, 57]. The canisters have some advantages in that they are reactive with few compounds and they prevent sample cross contamination much more effectively. However, the per unit cost alone makes them very impractical as disposable collection vessels in medical applications. Tedlar, on the other hand, has the significant advantage of being disposable.

It is questionable whether tedlar is the most suitable material for gas sampling. Though it is relatively inert, there is evidence of off gassing and adsorption of some volatile species to the bag walls. Tedlar bags release acetic acid and phenol and greatly adsorb indole and skatole, thereby having the potential to bias analysis. However, because a better material for gas sampling has not been proposed, Tedlar is still the material of choice.

4.1.4 Sampling Bag Reusability

Because exploratory experiments required many sample collections, we wanted to determine whether the breath collection bags could be cleaned and reused. The cleaning protocol for breath sampling bags revealed adequate cleaning of breath compounds and sample carryover within tolerable limits at less than 0.1%. It was determined that "uncleaned" breath compounds like Indole, Dodecane, DL-Limonene are also found in the ambient environment, making it questionable whether the observed compounds resulted from poor cleaning performance.

Additionally, an interesting observation is that the percent of VOCs from uncleaned bags falls within the normal variability observed as day-to-day variations (Table 10). Although it was not established as part of this thesis, the percent deviation of compounds for a single individual could result from other non-physiological sources such as imprecision in extraction conditions, storage stability, or instrumentation.

The scaling of figure 12 conceals another unexpected finding from this experiment. Comparison of new bags and cleaned bags reveals a reduction in contaminating peaks originating from tedlar material (Figure 26). This finding further supports the identification of tedlar as the source of phenol and acetemide, N,N-dimethyl and demonstrates that other background peaks come from other sources in collection and analysis. Therefore, with application of this cleaning protocol some background can be removed.

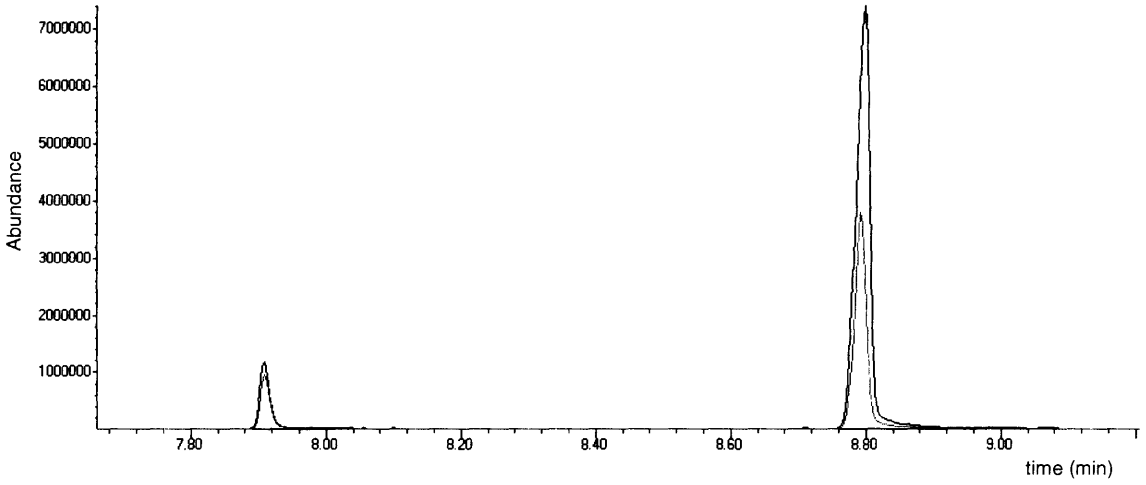

Figure 26: Reduction of phenol chromatographic peak from bag cleaning protocol. Phenol peak in new bag (black). Phenol peak in cleaned bag (blue).

4.1.5 Identification of Background

We identified a number of compounds that are not native to human exhaled breath but present in the breath signal nonetheless. It was important to identify these compounds initially so that's conclusions would not be based on their quantitation in remaining experiments. Several background components were found in large abundances and are identified in figure 12 and its supporting tables. Siloxane compounds such as cyclopentasiloxane, decamethyl are believed to arise due to bleed from the chromatographic column polymer, SPME fiber, and/or septa from either the GC inlet or sampling apparatus. Acetemide, N,N-dimethyl and phenol were previously attributed to the tedlar material.

Phenol, a constituent of the background signal, was used as a normalizing factor in quantitation (Section 2). It is important to acknowledge that area response correction by phenol is not a widely-used technique. Internal standards are normally used for this purpose. However, the evidence showing phenol as a consistent presence in Tedlar

bags was found in literature, and the results when corrected area response was applied were consistent with results reported by others in literature [28].

4.2 Method Development

4.2.1 SPME coating selection

One major drawback to SPME is that it necessarily excludes certain types of compounds in the breath matrix from analysis. This is minimized by having many available fiber coatings and choosing the one most responsive to breath. While CAR/PDMS would be the fiber of choice for its high affinity to the types of compounds in breath, evidence supporting the selection of PDMS/DVB highlights the importance of relying on separation and analysis experience for fiber coating selection.

The choice of PDMS/DVB as the optimal coating for the extraction of breath VOCs was determined according to which had selectivity for the greatest number of compounds and highest sensitivity. High selectivity was a reasonable aim since we desired a method to capture and analyze the widest breath profile possible. Because VOCs are found in nanomolar concentrations, choosing a coating with high sensitivity provided more complete extraction of poorly extracted VOCs and produced more prominent signals with improved signal-to-noise characteristics.

Nevertheless, two shortcomings of this approach can be identified. First, with only 120 targeted compounds for selectivity analysis, determination of the best coating for this purpose was based on a small fraction of the over 3000 previously identified VOCs in human breath. The 120 compounds were among the most frequently observed in past studies, and the analysis of selectivity was based on collections from the same

individual. So, while this work establishes superior selectivity with PDMS/DVB for a single subject, we did not necessarily establish the optimal coating for extraction of the entire diversity of compounds observed in breath.

Second, the 22 breath compounds targeted for sensitivity analysis represented further reduction of the 120 VOCs identified here. This was done intentionally as quantitative analysis for 120 VOCs would have required a much greater investment of time and resources. Efforts were made to establish that the 22 target compounds represented a diverse set of VOCs, but ultimately, selection of the targets was based on observation frequency and not physical or functional considerations. Moreover, the compounds most efficiently extracted by PDMS/DVB coating did not represent a wide diversity even from among the compounds selected. So, while sensitivity was established for many of the targeted compounds, it was not necessarily established whether PDMS/DVB would provide greater sensitivity to the diversity of compounds observed in breath. Alkanes, such as dodecane, tridecane, pentadecane, and undecane, were well extracted by the PDMS/DVB fiber, but overall the DVB/CAR/PDMS coating extracted these compounds with greatest efficiency. Carboxylic acids, sulfides, and the single ketone investigated were better extracted by the CAR/PDMS coating.

Additionally, the DVB/CAR/PDMS coating selected 36% of the 120 compounds versus 32% for PDMS/DVB making it a very close second choice for fiber. PDMS/DVB was selected because overall its sensitivity performance was preferred over the slightly superior selectivity found with DVB/CAR/PDMS. Compounds like β-Myrcene and 3-Carene for which PDMS/DVB demonstrated superior sensitivity were extracted 2.6x and 6.7x more effectively. Furthermore, this resulted in signal-to-noise improvements for these compounds of 1.8x and 3.3x, respectively.

4.2.2 Extraction Conditions

The profiles of the three observed extraction characteristics in Figure 13 demonstrate an important concept in solid phase microextraction: that for porous polymer coatings, competitive adsorption can cause analyte displacement and a reduction in extraction capacity for lower affinity compounds at equilibrium [58]

Ideally we would like to reach equilibrium between the breath sample matrix and the fiber. However, because we are looking at a complex matrix with many VOCs, definition of an appropriate adsorption time is difficult. Some analytes have high affinity to the fiber initially, but as time increases they may be competitively displaced reducing their concentration extracted on the fiber. This is may be an explanation for the response seen with by acetone and propanoic acid which decrease the longer extraction is held. When shorter than equilibrium extraction times are used, care must be taken to ensure that conditions are kept precisely constant for each sample. If compounds are not able to equilibrate, an increase in temperature or a decrease in time may have a significant effect on the relative amounts extracted from the sample. Compounds in the breath matrix were found to fall into 3 different categories. Those that do not reach equilibrium but continue to increase over the durations examined, those that reach equilibrium some time between 1 and 120 min, and those that were competitively selected out but reach equilibrium at amounts less than initially extracted. To find balance between these the conflicting equilibrium categories, an extraction time of 30 min was selected.

Another important parameter to optimize is extraction temperature. A preliminary investigation was carried out to explore the effect of extraction temperature. 37°C was preferred and chosen as the standard extraction temperature because of its

physiological significance as the temperature of gases in the alveoli along with the following reasons. Firstly, the higher temperature ensures the evaporation of condensed water from the Tedlar® bag surface. VOCs that would otherwise be trapped by the condensed water are released at this temperature leading to improved extraction [34]. Secondly, it was reasoned that higher temperatures than 37°C could potentially damage the bag material or cause more background volatiles to off gas from the bag wall.

Also important for the study of breath is that PDMS based coating do not concentrate major breath components like oxygen, carbon dioxide, or water vapor very well allowing more extraction capacity for VOCs in breath.

4.2.3 GC-MS Settings: resolution of pentane and isoprene

Multiple past studies have focused on single compounds as disease markers, but few have attempted to enumerate the many compounds in breath and test their fitness for disease detection. It is the goal of this project to develop a breath analysis technique that accounts for the great diversity in breath. However, because acute cardiac distress populations have been shown to produce higher levels of breath pentane and isoprene, it was of particular interest to monitor these markers in our study. The methods we developed were capable of detecting pentane and isoprene with match factors of 74 and 89, respectively. The VOCs were extracted by SPME and analyzed by mass spectroscopy, but AMDIS was required to deconvolve the signals in the resulting TIC, highlighting that separation with gas chromatography was still insufficient.

While we were able to achieve good separation between many of the chemicals present in breath with oven parameters, we were unable in the timeframe of this study to

establish a temperature program to separate pentane and isoprene from each other in our first round of studies. Quantitation on these compounds was difficult due to their coelution in chromatograms. Separation is more easily achieved with Poraplot Q and Poraplot U columns, but these columns are not of practical use for other VOCs in human breath. Figure 27 demonstrates how peaks in the TIC for pentane and isoprene merge into one using our current method. Peak splitting, seen with acetone in the figure, is common. However, separate ion components, representing two distinct compounds, are found in the mass spectra around the retention time of 1.14 min.

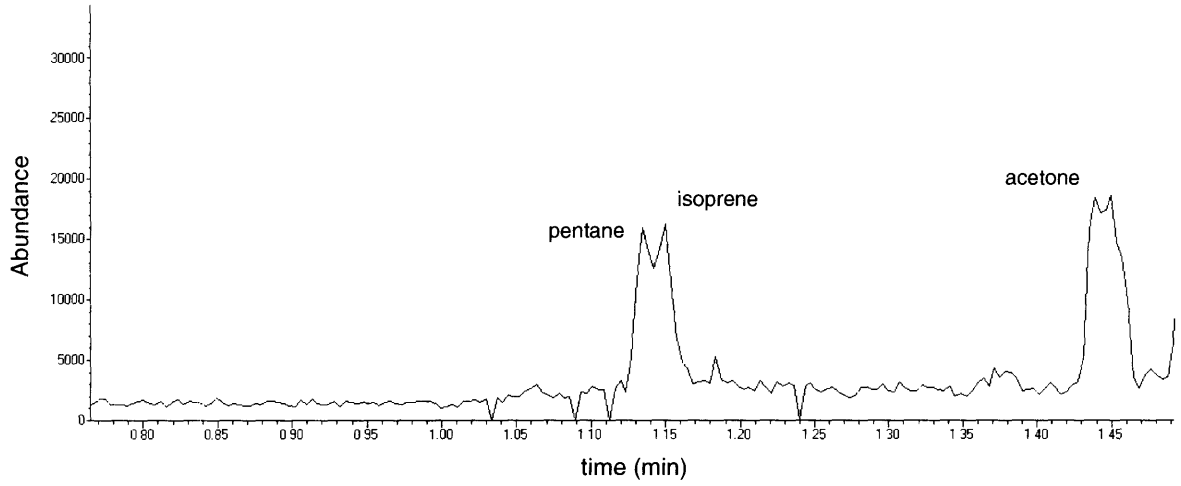

Figure 27: Coelution of pentane and isoprene in total ion chromatogram (TIC).

Although we were unsuccessful in resolving pentane and isoprene from one another in GC-MS analysis, it is possible that they will be well separated in GC-DMS analysis. Although component separation is primarily achieved by the GC, the nonlinearity of DMS provides an additional level of separation that may allow resolution of these two potentially important chemicals for disease detection. It was not possible to determine pentane and isoprene separation with GC-DMS in this study as differences in column length prevented correlation between GC-MS and GC-DMS spectra. However, this may be possible in future studies.

4.2.4 Sample Storage

As a first step to a field deployable device, samples obtained from the hospital will be stored before analysis in the laboratory. This will allow us to perform proof-of-concept experiments for biomarker determination. Recommended storage times vary in the literature. Conclusion regarding an appropriate storage time was not determined in this thesis. Previous studies of storage stability do not recommend storage beyond 6 to 9 hrs [34, 59]. Without experimental evidence otherwise, it is recommended that samples be stored for less than 9 hours. This poses a logistical problem for any sample collection that must be done in future trials. However, with careful planning, it is simple to overcome. Samples should not be collected unless arrangements can be made for analysis within the recommended storage time. It is imperative that once samples are collected that they be used in trials to determine possible biomarkers for disease. Therefore, proper communication must be maintained between study centers and the laboratory where analysis is to be performed. Limitations from storage instability may result in enrollment reduction in future studies as some candidates for breath analysis will not be considered due to timing constraints.

As noted in section 3.2.4, the trends from the storage stability study are suggestive. Significance was limited by high variability and underpowered analysis. Although results suggested sample instability at 24 hours with response changes of 29%, further studies should be performed to asses the storage stability of the sampling system. Storage durations greater than 9 hrs could greatly improve enrollment in future studies and permit faster determination of disease biomarkers.

4.3 Breath Characterization

The observed VOC variability in day-to-day experiments can be explained by two possible reasons. First, the large degree of variability could be evidence of considerable changes in breath composition from day to day. This has implications for future studies. If the normal variability is so significant, biomarkers for disease detection will have to produce changes above and beyond the normal variability in order to be identified and used in disease diagnosis. The power of pattern recognition as a data analysis tool, however, is to account for and eliminate normal variability seen across both diseased and healthy populations. Pattern recognition algorithms only determine fingerprints based on differences observed consistently in one group when compared to another regardless of the magnitude of those differences.

On the other hand, the observed variability could be caused by analytical imprecision. The breath profiles presented in Figure 16 identify two regions of extraction – an initial concentrating phase where analytes are filling active sites of the divinylbenzene and an equilibrium phase, where analyte concentrations remain stable. During the concentrating phase extracted quantities of each compound are changing and the rate can vary depending on polarity, molecular weight, and temperature. 30 min, an interval during the concentrating phase for many VOCs, was chosen for extraction primarily because it limited competitive displacement of compounds that had lower affinities for the active sites. However, if the timing of extraction is not precise, extraction times in the concentrating phase can lead to sizable changes in the quantities extracted and observed. For example, if extraction is held 5 min longer (an increase of 17%), undecane extraction could increase by approximately 2000 abundance units, nearly 60% of its observed amount at 30 min. Very small error suggests that timing imprecision did

not dramatically effect quantitation of undecane. However, this brief analysis demonstrates the importance of extraction precision for proper quantitation. It should also be noted that measurement errors for propanoic acid were not found to be nearly as small.

If we think of breath compounds as signal and background compounds as noise, than the numerical measurements evaluating single individual and inter-individual variability all suffered from very poor signal to noise ratios. PCA plots that were presumed to demonstrate variability probably resulted more from background artifact than from variation in breath signal. The primary reason for this response can be traced to fundamental principles underlying PCA and numerical measures of similarity. Each total ion chromatogram is treated as a vector of features. So, the magnitudes of the abundances at specific retention times are essentially multiplied. This assigns greater weight to higher abundance features particularly if they correlate to high abundance features in the compared chromatogram. What results is that background, instead of breath dominates PCA and the numerical functions applied in this study.

5 CONCLUSION

The research that makes up this thesis is part of a larger, multiphase study to determine the diagnostic potential of breath analysis. In this work, a method was developed for the analysis of human breath with Solid Phase Microextraction (SPME) and using both Gas Chromatography- Mass Spectrometry(GC-MS) and Gas Chromatography- Differential Mobility Spectrometry (GC-DMS) systems. The method was validated by techniques previously described in literature. Several parameters of the collection and analysis method were explored and optimized to meet the goals for the larger study. Specifically, the aim is to develop a stand alone device for medical diagnostics. Future work on bioinformatic modeling of the sensor output and biomarker discovery will also be needed for future clinical trials. The needs of this bioinformatic approach were highly considered in the development of a protocol for breath collection and sample analysis.

Breath is a complex compound mixture to analyze. From these studies, we determined that attempts to concentrate and analyze breath are confounded by the complex interactions of breath compounds with each other, and more importantly, with adsorptive polymers. SPME extraction methods were gleaned from the literature and optimized to concentrate trace levels of volatile organic compounds (VOCs) in breath. Part of the attractiveness of SPME for breath analysis is that it is simple enough to be deployed in field applications, which combined with DMS for fast analysis will allow breath analysis to make its greatest impact on medical diagnostics.

This project also dealt with the characterization of breath using GC-DMS and GC-MS systems. With GC-DMS, this research represented the first attempt to implement breath analysis using SPME and GC-DMS. Preliminary investigation of the resulting signals

demonstrated that a large signal can be obtained from these sample types greater deal of information than has previously been seen with DMS analyzed samples. The potential for disease detection from breath has not yet to been demonstrated although results presented here using PCA and distance functions do show promise.

Characterization of breath using GC-MS revealed similar breath compounds to previous research in the literature. Quantitative analysis demonstrated the need for rigorous control of collection and extraction parameters to obtain reproducible results. SPME extraction conditions, in particular, must be maintained within tight controls as slight changes can dramatically affect results.

Comparison of signal variability in single individuals and between multiple subjects revealed potential biases when PCA was applied. This highlighted the need for careful correction of background in the future. The background includes contaminants from the environment although it is sampling apparatus contamination that may be a significant artifact.

The protocol developed in this thesis provides a robust signal suited for diagnostic modeling, classification, and disease diagnosis. The combination of GC-MS and GC-DMS in future analysis will provide an opportunity to validate the origin of identified features of disease biomarkers. Solid phase microextraction, differential mobility spectrometry, and the application of classification algorithms to these sensor outputs have the potential to push the frontiers of breath analysis to a point that it may one day be realized in clinical application.

6 FUTURE DIRECTIONS

6.1 Removal of Background Contamination

In several sections of this thesis, background signals have been identified as important. In the case of quantitation, background signals from phenol were used for normalization. However, no other advantages to having such prominent background signals were identified. Disadvantages include possible coelution with breath compounds and peak tailing in the chromatogram leading to diminished resolution, higher noise levels as characteristic masses are consistently found throughout the entire chromatogram, and most notably background domination in numerical calculations.

Future efforts should be devoted to reducing background signals, and this study has highlighted area to focus those efforts:

1) Phenol and Acetemide, N,N-dimethyl originate from Tedlar sampling bags. Heating and purging with nitrogen gas was shown to reduce their presence. While the adsorption temperature was chosen for physiological reasons, lower temperatures should be investigate to determine if there is a balance between improvement in extraction characteristics at higher temperatures and improvement in background contamination levels at lower temperature. Complete reduction could also be achieved by exploring other sampling options including alternative sampling vessels and direct to SPME sampling.

2) The siloxane based contaminates observed actually originate from septa and polymer coatings on SPME fibers and chromatographic columns. While it is difficult to reduce signal contamination due to column bleed, fiber contamination can be limited by exploring lower desorption temperatures. Care must be taken, to ensure that high flow in

the inlet permits fast desorption times and burst delivery of compounds on to the column. Lower desorption temperatures should also improve coating lifetime.

The GC inlet used in this study contained a prepunctured septum which limits contamination. However, the septum fitting on tedlar bags represented a potentially significant source of siloxane contamination. The needle tip of SPME holders can core the septum when it is introduced into the collection bag. While large piece of septum do not get into the SPME assembly, small particles can and may lead to contamination. Future investigators that use Tedlar bags should investigate other septum materials.

6.2 Field Deployable Device

This work represented the initial phase of project to develop a standalone device for disease detection. Before a field deployable device can be produce additional technical considerations will need to be figured out. How will sampling and separation be done? Recent work has demonstrated the feasibility of on-site SPME sampling, eliminating the need for sample storage or transportation. Coupled with portable gas chromatography and differential mobility spectrometers, this system has the potential to realize the portability and convenience objectives that will make breath analysis clinically applicable. Refinement to the method established in this thesis would be required to optimize performance. Additionally, direct to fiber sampling with fast extraction times should be explored for sample concentration. At fast extraction times and high sample flow rates, analytes are efficiently adsorbed, minimizing competitive displacement, and improving extraction precision.

REFERENCES

1. Phillips, M., *Breath tests in medicine.* Scientific American, 1992. **267**(1): p. 74-79.
2. Zarling, E. and M. Clapper, *Technique for gas-chromatographic measurement of volatile alkanes from single-breath samples.* Clinical Chemistry, 1987. **33**(1): p. 140-141.
3. Miekisch, W., J. Schubert, and G. Noeldge-Schomburg, *Diagnostic potential of breath analysis-focus on volatile organic compounds.* Clinica Chimica Acta; International Journal of Clinical Chemistry, 2004. **347**(1-2): p. 25-39.
4. Weitz, Z., et al., *High breath pentane concentrations during acute myocardial infarction.* Lancet, 1991. **337**(8747): p. 933-5.
5. Mendis, S., et al., *Breath pentane and plasma lipid peroxides in ischemic heart disease.* Free Radical Biology & Medicine, 1995. **19**(5): p. 679-684.
6. Sobotka, P., et al., *Elevated breath pentane in heart failure reduced by free radical scavenger.* Free Radical Biology & Medicine, 1993. **14**(6): p. 643-647.
7. Springfield, J. and M. Levitt, *Pitfalls in the use of breath pentane measurements to assess lipid peroxidation.* Journal of Lipid Research, 1994. **35**(8): p. 1497-1504.
8. McGrath, L., R. Patrick, and B. Silke, *Breath isoprene in patients with heart failure.* European Journal of Heart Failure, 2001. **3**(4): p. 423-427.
9. Risby, T. and S. Sehnert, *Clinical application of breath biomarkers of oxidative stress status.* Free Radical Biology & Medicine, 1999. **27**(11-12): p. 1182-1192.
10. Kneepkens, C., G. Lepage, and C. Roy, *The potential of the hydrocarbon breath test as a measure of lipid peroxidation.* Free Radical Biology & Medicine, 1994. **17**(2): p. 127-160.
11. Kupari, M., et al., *Breath acetone in congestive heart failure.* The American Journal of Cardiology, 1995. **76**(14): p. 1076-1078.
12. Kaji, H., et al., *Evaluation of volatile sulfur compounds in the expired alveolar gas in patients with liver cirrhosis.* Clinica Chimica Acta; International Journal of Clinical Chemistry, 1978. **85**(3): p. 279-284.
13. Rooth, G. and S. Ostenson, *Acetone in alveolar air, and the control of diabetes.* Lancet, 1966. **2**(7473): p. 1102-1105.
14. Bogen, E., *Drunkenness -- a quantitative study of acute alcoholic intoxication.* JAMA: The Journal of the American Medical Association, 1927. **89**: p. 1508-1511.
15. Harger, R., R. Forney, and H. Barnes, *Estimation of the level of blood alcohol from analysis of breath.* The Journal of Laboratory and Clinical Medicine, 1950. **36**(2): p. 306-318.
16. Borkenstein, R. and H. Smith, *The Breathalyzer and its application.* Medicine, Science, and the Law, 1961. **2**: p. 13-22.
17. Hlastala, M., *The alcohol breath test - a review.* Journal of Applied Physiology, 1998. **84**(2): p. 401-8.
18. Gustafsson, L., *Exhaled nitric oxide as a marker in asthma.* The European Respiratory Journal. Supplement., 1998. **26**: p. 49S-52S.
19. Chapman, J. and A. Choi, *Exhaled monoxides as a pulmonary function test: use of exhaled nitric oxide and carbon monoxide.* Clinics in Chest Medicine, 2001. **22**(4): p. 817-836.

20. Kharitonov, S. and P. Barnes, *Biomarkers of some pulmonary diseases in exhaled breath.* Biomarkers, 2002. **7**(1): p. 1-32.
21. Berne, R., et al., *Physiology.* 5th ed. 2004, St. Louis: Mosby.
22. Manolis, A., *The diagnostic potential of breath analysis.* Clinical Chemistry, 1983. **29**(1): p. 5-15.
23. Pauling, L., et al. *Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography.* in *Proceedings of the National Academy of Sciences of the United States of America.* 1971.
24. Phillips, M., et al., *Variation in volatile organic compounds in the breath of normal humans.* Journal of Chromatography. B, Biomedical Sciences and Applications, 1999. **729**(1-2): p. 75-88.
25. Stone, B., et al., *Effect of regulating cholesterol biosynthesis on breath isoprene excretion in men.* Lipids, 1993. **28**(8): p. 705-708.
26. Stewart, R. and E. Boettner, *Expired-air acetone in diabetes mellitus.* New England Journal of Medicine, 1964. **270**: p. 1035-1038.
27. Van Gossum, A. and J. Decuyper, *Breath alkanes as an index of lipid peroxidation.* The European RespiratoryJournal, 1989. **2**(8): p. 787-791.
28. Grote, C. and J. Pawliszyn, *Solid-phase microextraction for the analysis of human breath.* Analytical Chemistry, 1997. **69**(4): p. 587-96.
29. Krotoszynski, B., G. Gabriel, and H. O'Neil, *Characterization of human expired air: a promising investigative and diagnostic technique.* Journal of Chromatographic Science, 1977. **15**(7): p. 239-244.
30. Mendis, S., P. Sobotka, and D. Euler, *Pentane and isoprene in expired air from humans: gas-chromatographic analysis of single breath.* Clinical Chemistry, 1994. **40**(8): p. 1485-1488.
31. Phillips, M., *Method for the Collection and Assay of Volatile Organic Compounds in Breath.* Analytical Biochemistry, 1997. **247**(2): p. 272-278.
32. Ochiai, N., et al., *Analysis of volatile sulphur compounds in breath by gas chromatography-mass spectrometry using a three-stage cryogenic trapping preconcentration system.* Journal of Chromatography. B, Biomedical Sciences and Applications, 2001. **762**(1): p. 67-75.
33. Boyd-Boland, A. and J. Pawliszyn, *Solid-Phase Microextraction Coupled with High-Performance Liquid Chromatography for the Determination of Alkylphenol Ethoxylate Surfactants in Water.* Analytical Chemistry, 1996. **68**(9): p. 1521-1529.
34. Hyspler, R., et al., *Determination of isoprene in human expired breath using solid-phase microextraction and gas chromatography-mass spectrometry.* Journal of Chromatography. B, Biomedical Sciences and Applications, 2000. **739**(1): p. 183-90.
35. Miller, R.A., et al. *A MEMS Radio-Frequency Ion Mobility Spectrometer for Chemical Agent Detection.* in *Solid-State Sensor and Actuator Workshop.* 2000. Hilton Head Island, SC.
36. Miller, R.A., et al., *A novel micromachined high-field asymmetric waveform-ion mobility spectrometer.* Sensors and Actuators, 2000. **67**: p. 300-6.
37. Miller, R.A., et al., *A MEMS radio-frequency ion mobility spectrometer for chemical vapor detection.* Sensors and Actuators, 2001. **91**: p. 307-18.
38. Eiceman, G.A., et al., *Separation of ions from explosives in differential mobility spectrometry by vapor-modified drift gas.* Analytical Chemistry, 2004. **76**(17): p. 4937-4944.
39. Eiceman, G.A., et al., *Miniature radio-frequency mobility analyzer as a gas chromatographic detector for oxygen-containing volatile organic compounds,*

pheromones, and other inset attractants. Journal of Chromatography, 2001(917): p. 205-17.
40. Krylov, E.N., et al., *Field Dependence of Mobilities for Gas-Phase-Protonated Monomers and Proton-Bound Dimers of Ketones by Planar Field Asymmetric Waveform Ion Mobility Spectrometer (PFAIMS).* The Journal of Physical Chemistry. A, Molecules, Spectroscopy, Kinetics, Environment & General theory, 2002. **106**(22): p. 5437-44.
41. Shnayderman, M., et al., *Species-specific bacteriaidentification using differential mobility spectrometry and bioinformatics pattern recognition.* in Press, Analytical Chemistry, 2005.
42. Krebs, M.D., et al., *Detection of Biological and Chemical Agents using Differential Mobility Spectrometry (DMS) Technology.* IEEE Sensors Journal, 2005.
43. Mason, E. and E. McDaniel, *Transport Properties of Ions in Gases.* 1988, New York: Wiley.
44. Guevremont, R., *High-field asymmetric waveform ion mobility spectrometry: a new tool for mass spectrometry.* Journal of Chromatography. A, 2004. **1058**(1-2): p. 3-19.
45. Schmidt, H., et al., *Microfabricated differential mobility spectrometry with pyrolysis gas chromatography for chemical characterization of bacteria.* Analytical Chemistry, 2004. **76**(17): p. 5208-5217.
46. Merrick, W., et al., *Monitoring drinking water quality using pyrolysis-differential mobility spectrometry.* In Review - Rapid Communications in Mass Spectroscopy, 2005.
47. Deng, C., et al., *Determination of acetone in human breath by gas chromatography-mass spectrometry and solid-phase microextraction with on-fiber derivatization.* Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 2004. **810**(2): p. 269-75.
48. Spinhirne, J., J. Koziel, and N. Chirase, *Sampling and analysis of volatile organic compounds in bovine breath by solid-phase microextraction and gas chromatography-mass spectrometry.* Journal of Chromatography, A, 2004. **1025**(1): p. 63-9.
49. Schubert, J., et al., *C02-controlled sampling of alveolar gas in mechanically ventilated patients.* Journal of Applied Physiology, 2001. **90**: p. 482-492.
50. Miekisch, W., J. Schubert, and G. Noeldge-Schomburg, *Analysis of volatile disease markers in blood.* Clinical Chemistry, 2001. **47**(6): p. 1053-1060.
51. Levitt, M., C. Ellis, and J. Furne, *Influence of method of alveolar air collection on results of breath tests.* Digestive Diseases and Sciences, 1998. **43**(9): p. 1938-45.
52. Spinhirne, J., J. Koziel, and N. Chirase, *A device for non-invasive on-site sampling of cattle breath with solid-phase microextraction.* Biosystems Engineering, 2003. **84**(2): p. 239-46.
53. Brereton, R., *Chemometrics: data analysis for the laboratory and chemical plant.* 2003, West Sussex: Wiley and sons,Ltd.
54. Stein, S., *An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectroscopy data.* Journal of the American Society for Mass Spectrometry, 1999. **10**: p. 770-781.
55. Phillips, M. and J. Greenberg, *Ion-trap detection of volatile organic compounds in alveolar breath.* Clinical Chemistry, 1992. **38**(1): p. 60-65.
56. Davies, T., *The new automated mass spectrometry deconvolution and identification system (AMDIS).* Spectroscopy Europe, 1998. **10**(3): p. 24-27.

57. Pleil, J.D. and A.B. Lindstrom, *Measurement of volatile organic compounds in exhaled breath as collected in evacuated electropolished canisters.* Journal of Chromatography. B, Biomedical Applications, 1995. **655**: p. 271-9.
58. Pawliszyn, J., *Solid Phase Microextraction: Theory and Practice*. 1997, New York: Wiley-VCH.
59. Lee, J., et al., *Determination of Volatile Organic Compounds (VOCs) Using Tedlar Bag/Solid-phase Microextraction/Gas Chromatography/Mass Spectrometry (SPME/GC/MS) in Ambient and Workplace Air.* Bulletin of the Korean Chemical Society, 2002. **23**(3): p. 488-495.